Immunomodulatory properties of probiotic bacteria

Effects on dendritic cells and their interactions with NK cells and T cells

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Preface

The work included in the present Ph.D. thesis has been conducted in the Nutritional Immunology Group at BioCentrum-DTU, Technical University of Denmark between September 2003 and February 2007.

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In spring 2005 I spent five memorable months at the National Cancer Research Institute in Genoa, Italy, with Barbara Morandi, Antonella D'Agostino, Paolo Carrega, and Gabriele Lui in Guido Ferlazzo's group and all the other amazing people of the lab. Thanks, Barbara for sharing your friends and family with me, and Guido, I cannot describe how welcome I felt, I learned a lot and I hope we will continue working together. The Italian connection also meant that Irene Bonaccorsi from Sicily came to work in Denmark for a while, which was a great experience.

Most importantly, I wish to thank my family and friends for lending their ears to all the talk about good and bad bugs and for keeping my spirits up. Finally, Thomas, thank you so much for always being there for me, I would never had made it without your love and understanding.

Abbreviations

AD	atopic dermatitis
APC	antigen-presenting cell(s)
B.	*Bifidobacterium*
BDC	blood myeloid dendritic cell(s)
BMDC	bonemarrow-derived dendritic cell(s)
CBA	cytometric bead array
CCR	chemotactic cytokine receptor
CD	cluster of differentiation
cpm	counts per minute
DC	dendritic cell(s)
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing nonintegrin
E.	*Escherichia*
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin units
FACS	flow assisted cell sorting
FCS	foetal calf serum
GALT	gut-associated lymphoid tissue
GM-CSF	granulocyte macrophage colony-stimulating factor
Flt3-L	FMS-like tyrosine kinase 3 ligand
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
iDC	immature dendritic cell(s)
IFN	interferon
IFR	interfollicular region
Ig	immunoglobulin
IL	interleukin
KIR	killer immunoglobulin-like receptor
L.	*Lactobacillus*
LAB	lactic acid bacteria
LP	lamina propria
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MACS	magnetic activated cell sorting
M cell	microfold cell
mDC	mature dendritic cell(s)
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
Mo	monocyte(s)
MoDC	monocyte-derived dendritic cell(s)
NCR	natural cytotoxicity receptor
NFκB	nuclear factor κB
NK cell	natural killer cell
NKT cell	natural killer T cell
Nod	nucleotide-binding oligomerisation domain
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell(s)
PE	phycoerythrin
PG	peptidoglycan
PP	Peyer's patches
PRR	pattern recognition receptor
SD	standard deviation
SED	subepithelial dome region
SN	supernatant
Tc	cytotoxic T cell
TCR	T cell receptor
TGF	tumour growth factor
Th	helper T cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin

Dansk resumé

Visse mælkesyrebakterier, som udgør en del af tarmfloraen, betragtes som gavnlige idet de konkurrerer med patogene bakterier om at adhærere til tarmepitelet og nedbryder ufordøjelige fødevarekomponenter. En anden vigtig egenskab ved disse såkaldt probiotiske bakterier er, at de påvirker immunsystemet. Denne afhandling beskriver, hvordan udvalgte tarm-afledte bakteriestammer påvirker forskellige antigen-præsenterede celler, og hvordan natural killer (NK) celle- og T-cellerespons påvirkes af bakterielt stimulerede antigen-præsenterende celler.

Autologe NK-celler og dendritiske celler (DC) aktiverer gensidigt hinanden, og denne interaktion menes at have betydning for NK-cellernes cytotoksiske aktivitet mod kræftceller og for T-celle-polarisering. Det første studie i denne afhandling slår fast, at mælkesyrebakterier, i kraft af deres effekt på monocyt-afledte DC, kan påvirke NK-cellerespons. Alle de undersøgte mælkesyrebakterier øgede NK-cellers proliferation og cytotoksiske aktivitet via modning af DC, men kun IL-12-inducerende mælkesyre-bakterier inducerede IFN-γ-produktion i NK-celler. Udvalgte mælkesyrebakterier, som er i stand til at inhibere IL-12-produktion i DC, inhiberede også IFN-γ-produktionen i NK-celler.

Efterfølgende blev det undersøgt, hvorvidt den stamme-afhængige induktion af IL-12, som mælkesyrebakterier og *E. coli*-stammer giver anledning til i monocyt-afledte DC, også finder sted i DC og monocytter oprenset direkte fra blod. Begge slags antigen-præsenterende celler fra blod producerede cytokiner, når de blev stimuleret med bakterier, og den opnåede cytokin-profil for hver bakterie lignede mønstret induceret i monocyt-afledte DC, med undtagelse af TNF-α og IL-6, som ikke blev induceret af de samme bakterier i blod-DC/monocytter og i monocyt-afledte DC. Autologe NK-celler producerede IFN-γ, når de blev stimuleret med blod-DC, monocytter eller monocyt-afledte DC samt IL-12-inducerende bakterier, mens kun DC inducerede et kraftigt IFN-γ-respons i allogene T-celler.

In vitro-genererede DC er en meget brugt model for vævs-DC, men de adskiller sig på visse punkter fra tarm-DC, som er i kontakt med tarmfloraen. I det sidste studie isolerede vi DC fra Peyer's patches, mesenterielle lymfeknuder og milt fra mus og stimulerede dem med mælkesyrebakterier og *E. coli*. DC fra milt og mesenterielle lymfeknuder reagerede på stimulering med bakterier med en cytokinproduktion, som var sammenlignelig med responset i in vitro-genererede DC. DC fra Peyer's patches producerede kun IL-6. Celler fra milt og mesenterielle lymfeknuder producerede IFN-γ, når de blev stimuleret med de bakterier, som inducerer IFN-γ-produktion i NK- og T-celler via in vitro-genererede DC. Især celler fra de mesenterielle lymfeknuder producerede store mængder IFN-γ, hvilket kan betyde at lymfeknude-NK-celler potentielt kan producere meget cytokin, når de udsættes for tarmbakterier.

Abstract

Certain lactic acid bacteria (LAB) are part of the commensal intestinal flora and considered beneficial for health, as they compete with pathogens for adhesion sites in the intestine and ferment otherwise indigestible compounds. Another important property of these so-called probiotic bacteria is the ability to modulate the immune response. This thesis describes the immunomodulatory properties of gut-derived bacterial strains on different antigen-presenting cells, and the effector cell responses elicited by bacterially stimulated antigen-presenting cells in natural killer (NK) cells and T cells.

Autologous NK cells and mature dendritic cells (DC) mutually activate each other and this interaction is believed to be important for NK cytotoxic activity against cancer cells and for T cell polarisation. The first study included in this thesis establishes that LAB, as potent stimulators of monocyte-derived DC, are capable of directing NK cell responses. All tested strains increased NK cell proliferation and cytotoxic activity via maturation of DC, whereas only IL-12-inducing LAB induced IFN-γ production in NK cells. Specific LAB, capable of inhibiting IL-12 production in DC also inhibited IFN-γ production in NK cells.

Secondly, it was investigated whether the strain-dependent induction of IL-12 by LAB and *E. coli* strains observed in monocyte-derived DC also occurred in freshly isolated blood myeloid DC and monocytes. Both types of blood antigen-presenting cells produced cytokines when stimulated with bacteria, and the cytokine pattern induced by specific bacteria resembled the pattern induced in MoDC, except for TNF-α and IL-6, which were induced in response to different bacteria in blood DC/monocytes and monocyte-derived DC. Autologous NK cells produced IFN-γ when cultured with blood DC, monocytes and monocyte-derived DC and IL-12-inducing bacteria, whereas only DC induced IFN-γ production in allogeneic T cells.

In vitro-generated DC is a commonly used model of tissue DC, but they differ in certain aspects from intestinal DC, which are in direct contact with the intestinal microbiota. In the last study, we isolated DC from Peyer's patches, mesenteric lymph nodes, and spleens of mice, and stimulated these cells with strains of LAB and *E. coli*. Spleen and mesenteric lymph node DC responded to stimulation with cytokine production comparable to in vitro-generated DC. Peyer's patch DC produced only IL-6. Cells from spleen and mesenteric lymph nodes enriched in DC rapidly produced IFN-γ when stimulated with the bacteria that induce IFN-γ production in NK and T cells via in vitro-generated DC. Especially mesenteric lymph node cells produced large amounts of IFN-γ, which may indicate that mesenteric lymph node NK cells have a strong potential for cytokine-production in response to commensal bacteria.

Table of content

1. Introduction

The aim of this chapter is to introduce the reader to the immune system, in particular cells of the innate immune system: dendritic cells (DC) and natural killer (NK) cells, as well as the gut-associated lymphoid tissue. In addition, an introduction to the gastro-intestinal flora and to probiotics is given, with particular emphasis on the *interactions between immune cells and intestinal bacteria*.

The immune system – immunity and tolerance

The mammalian immune system has developed concomitantly with surrounding organisms to offer optimal defence against pathogens while preserving self-tolerance. Cells of the immune system are equipped with an array of receptors of varying complexity, as the primary defence against pathogens relies on detection of pathogen-associated molecular patterns and of specific peptide epitopes. Not only pathogens are detected by the immune system; also the microorganisms that populate skin and mucosal surfaces are recognised and contribute to the development of a balanced immune system. Tolerance against self-antigens, commensal microorganisms, and harmless antigens can be imperfect, which results in autoimmune disease and allergies. The incidence of these diseases is on the rise in Western countries and may reflect lifestyle-related changes affecting the regulation of the immune system. In this part, a brief overview of the immune system will be given focusing on the role of DC and NK cells, the cross-talk between these cell subsets and the effect on T cell polarisation. In addition, the gut-associated lymphoid tissues (GALT) will be introduced to enable discussion of the interaction of the immune system with commensal bacteria.

Cells of the immune system

The immune system consists of humoral factors, including antibodies and complement proteins, as well as a number of cell types of haematopoietic origin. The cells of the immune system originate in the bonemarrow and differentiate in the bonemarrow or in other tissues. T cells are characterised by the T cell receptor (TCR) recognising clone-specific peptides. T cells are subdivided into $CD8^+$ T cells, which are *cytolytic* (Tc cells) against cells displaying their specific peptide on MHC class I molecules, and $CD4^+$ T cells, which provide *help* (Th cells) for Tc cells and for B cells by the means of cell-to-cell contact and cytokine secretion. B cells have antigen-specific receptors, immunoglobulins that resemble antibody molecules. When B cells bind antigen and receive Th cell help they produce and secrete antibodies against the antigen. Thus, Tc cells are effective against intracellular pathogens, whereas B cell antibodies

are required to combat extracellular pathogens. For T cells to be activated they have to encounter an antigen-presenting cell (APC), presenting peptides derived from an antigen on surface MHC molecules.

So-called professional APC are monocytes (Mo), macrophages, B cells and DC that all have phagocytic capacities, ingest and process extracellular antigen, and expose peptides on MHC class II. These APC and all other cells of the body can also expose peptides derived from intracellular antigens or from self-proteins on MHC class I. Three signals are considered important in the communication between Th cells and APC: Firstly, the binding of MHC II to the TCR and CD4, secondly the binding of CD28 on the T cell to either CD80 (B7.1) or CD86 (B7.2) on the APC and finally stimuli derived from the APC or the environment (cytokines, chemokines and other soluble factors).

T cells and B cells derive from common lymphoid progenitor cells, which are shared with NK cells and natural killer T cells (NKT cells). Monocytes and DC (with one exception, the plasmacytoid DC) derive from myeloid progenitor cells. Another much employed subdivision of the immune system refers to the specificity of recognition. B and T cells are cells of the adaptive immune system, as they possess a multitude of highly specific receptors, and this feature enables repeated recognition of the same antigen, i.e. immunological memory. NK cells, NKT cells, Mo/macrophages, and DC possess receptors that are less specific, such as Fc receptors and Toll-like receptors (TLRs). These cell types are together with the complement system considered part of the non-specific or innate immune system, which is comparable to the immune system of invertebrates. However, as is clear from the T cell-APC interactions, the adaptive and the innate immune system rarely function independently of each other.

Dendritic cells - the professionals of antigen presentation and T cell polarisation

DC derive their name from long dendrites or veils protruding from the cell body, which enable the cell to interact closely with antigen and with other cells. These two situations describe the main roles of DC: antigen-uptake and antigen-presentation. Non-stimulated or “immature” DC circulate in the blood and migrate to tissues and lymph nodes. Here, DC are highly phagocytic and continuously sample the environment for soluble antigens, particles and apoptotic cells. After taking up antigen they reduce phagocytosis and migrate to the T cell areas of lymph nodes or the spleen. Simultaneously, DC upregulate MHC II molecules for antigen presentation as well as ligands for CD28 and other co-stimulatory molecules, and start cytokine secretion to provide T cells with the three stimuli needed to elicit a specific T cell response against the antigen. Because DC deliver all three signals, this is the only cell type capable of presenting antigen to naïve T cells. When memory T cells encounter their specific antigen, less stimulation is needed to initiate a response. The process changing the DC phenotype from phagocytic cell to T cell stimulatory cell is termed “maturation”. Mature DC express high levels of MHC I

and II, CD80, CD86, CD40, CD83 and CCR7, the latter being the chemokine receptor governing migration towards T cell areas of lymph nodes. During the course of an immune response, so-called pro-inflammatory mediators are released (TNF-α, IL-1β, IL-6, PGE_2), which can also mature DC and prepare them for interaction with T cells [1].

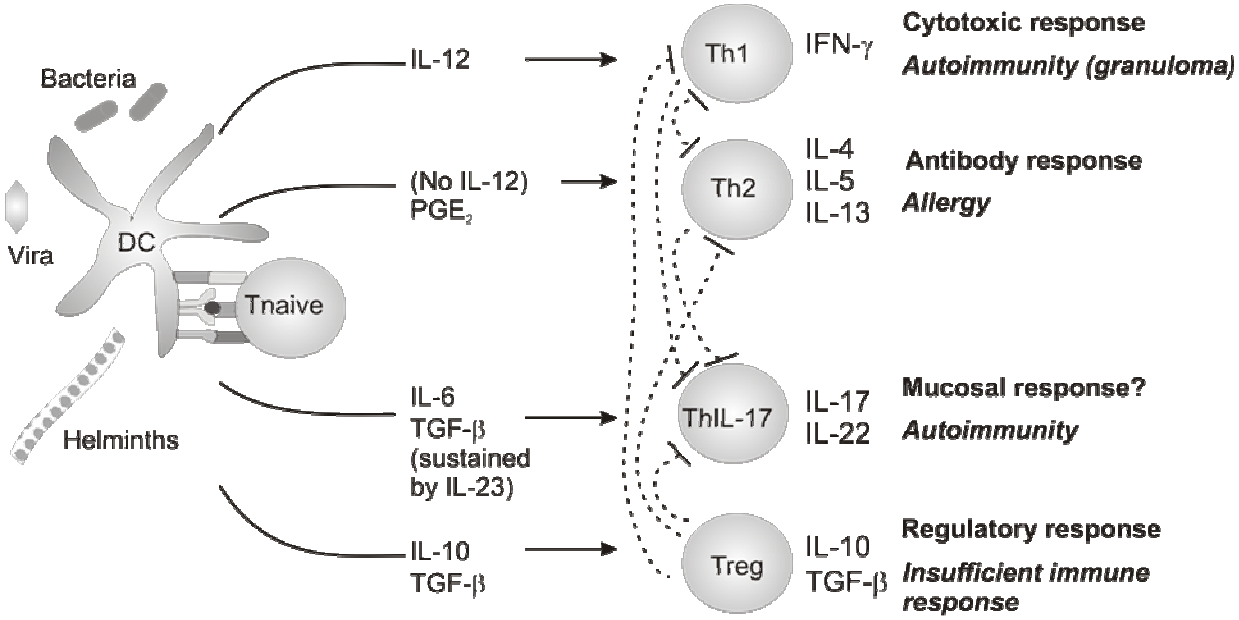

Figure 1.1: Polarisation of naïve T helper cells by DC secreting specific cytokines. DC secrete cytokines and upregulate MHC and co-stimulatory molecules in response to stimuli from microbes, apoptotic cells and other cells. The nature of T helper cell responses is governed by the cell-to-cell contact between DC and naïve T cells and by the combinations of DC-derived cytokines. Different subsets of T helper cells mutually inhibit the development of other subsets. Arrows indicate *induction*, broken lines indicate *inhibition.* Modified from Romagnani [2].

Th cell responses against soluble antigens and particulate antigens, such as bacteria and apoptotic cells, are very diverse, and this segregation of responses is mainly due to the ability of the different antigens to stimulate upregulation of co-stimulatory molecules and production of specific cytokines by DC. The diversification of Th cell responses, which result in the promotion of B cell, Tc cell, ThIL-17 cell or regulatory T cell responses is termed 'polarisation', because T cells promoting one type of response suppress the other responses [2]. Th cell polarisation by DC is illustrated in Figure 1.1. Th1 cell responses are essential for mounting Tc responses against intracellular pathogens, but may also promote autoimmune granuloma formation [2]. Th2 cells promote plasma B cell development essential for an antibody response against extracellular pathogens including helminths and soluble antigens such as toxins, including class-switching to IgE, and a Th2-skewed response may therefore give a predisposition to develop allergies [2]. Different subsets of regulatory T cells have been described, of which some

(naturally occurring $CD4^+CD25^+$ T cells) originate in the thymus, and others (inducible $CD4^+CD25^+$ T cells, Tr1 and Th3 cells) can be induced in a cytokine milieu dominated by IL-10 and TGF-β [3, 4]. Finally, ThIL-17 cells seem to develop in the presence of TGF-β, if also IL-6 is present, and are maintained by IL-23. These cells have been found in autoimmune lesions, implicating that they have a role in uncontrolled inflammation, but they may also have a beneficial role combating extracellular pathogens as has been described in the intestine [5].

In addition to the DC described above, which have the characteristics of myeloid cells and express the integrin CD11c both in mice and in humans, a subset of DC exists with poorer antigen-presenting properties, but with a large potential to secrete type 1 interferons (IFN-α and IFN-β). These cells, presumably derived from the lymphoid lineage, are designated plasmacytoid DC (pDC), and they are especially effective in the protection against vira. Human pDC express CD123 but not CD11c [6]. It should be noted, however, that there may be plasticity between DC of the two lineages, and recently it has been proposed that myeloid and plasmacytoid DC can develop from the same precursor under the influence of different cytokines [7]. Myeloid and plasmacytoid DC express different repertoires of TLRs, enabling detection of different pathogens (Table 1.1). TLRs are a family of receptors sharing the same intracellular signalling pathways, but recognising different conserved structures on microorganisms. TLRs are predominantly expressed by the cells of the innate immune system, enabling rapid initiation of an immune response towards invading microorganisms. Myeloid DC are unique among immune cells in that they respond to the majority of TLR ligands [8].

Table 1.1: Toll-like receptors (TLRs), TLR ligands, the presence of ligands in microorganisms, and TLR expression on subsets of DC [9, 10].

TLR	Ligand	Microorganism	Myeloid DC	Plasmacytoid DC
1	Triacyl lipopeptides	Bacteria	+	+
2	Lipoprotein, lipoteichoic acid, peptidoglycan, zymosan	Gram-positive bacteria, yeasts	+	+ / -
3	Double stranded RNA, poly(I:C)	Virus	+	+ / -
4	Lipopolysaccharide, mannans	Gram-negative bacteria, fungi	+	-
5	Flagellin	Flagellated bacteria	+	+ (mouse)/ - (human)
6	Diacyl lipopeptides	Bacteria	+	+
7	Single stranded RNA, imidazoquinolines (R-848)	Virus	+	+
8	Single stranded RNA, R-848	Virus	+	+ (mouse)/ - (human)
9	Non-methylated CpG DNA	Bacteria, protozoa, virus	+ (mouse)/ - (human)	+
10	Orphan	?	+ (human only)	+ / - (human only)
11	Components of uropathogenic bacteria, profillin	Uropathogenic bacteria, protozoa	+ (mouse only)	?

Natural killer cells – controllers of "missing self"

NK cells were originally described as lymphocytes exerting spontaneous killing activity in the absence of antigen-specific interaction, as opposed to $CD8^+$ Tc cells [11]. They are characterised by the expression of CD56 and absence of CD3 in humans and by one or both of the markers NKR-P1 (CD161) or CD49b in mice [12]. In humans, NK cells express different levels of CD56. $CD56^{dim}$ NK cells co-express CD16, they are cytolytic and constitute 90% of NK cells in the blood. The $CD56^{bright}$ subset dominates in lymph nodes and these cells are competent cytokine producers. Both subsets are presumably also recruited to inflamed tissues [13]. In addition, NK cell-like cells have been identified in the intestine [14, 15]. In mice, a marker permitting similar distinction between a cytokine-producing and a cytolytic NK cell subset has not yet been identified.

Table 1.2: Main natural killer cell receptors controlling cytotoxicity in humans [16-18].

Function	Receptor	Ligand	Ligand-carrier cells (non-exhaustive list)
Activating	NKp30	?	Myeloid DC, tumour cells
	NKp44	?	Tumour cells
	NKp46	?	Tumour cells
	NKp80	Activation-induced C-type lectin (AICL)	Monocytes, lymphocytes, granulocytes
	DNAX accessory molecule (DNAM)-1	Nectin-2, Poliovirus receptor (PVR)	Myeloid DC
	NKG2D	UL16 binding proteins (ULBPs), Major histocompatibility complex class I chain-related (MIC)-A and MICB	Stressed cells, tumour cells
	CD16	Fcγ portion of antibodies	Antibody-coated cells
	CD59	?	Tumour cells
	CD69	?	Tumour cells
	Killer-cell immunoglobulin-like receptors (KIRs)	HLA-C, G and unknown ligands	?
Activating or inhibitory	2B4	CD48	DC, bone-marrow cells
	NK-T-B antigen (NTBA)	NTBA	NK and T cells
	NKR-P1	Lectin-like transcript 1	Activated leukocytes
Inhibitory	Killer-cell immunoglobulin-like receptors (KIRs)	HLA-A, B and C	Normal cells
	CD94/NKG2A	HLA-E (non-classical HLA class I molecule presenting fragments of other HLA class I molecules)	Normal cells
	?	4Ig-B7-H3	Neuroblastoma cells

Today it is known that the cytolytic activity of NK cells is tightly regulated by both inhibitory NK cell receptors, including the majority of Killer immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in mice, which recognise HLA class I molecules on target cells, and activating receptors,

whose known ligands are induced by cellular stress and other yet unknown factors (Table 1.2). When activating receptors of NK cells are engaged by target cells in the absence of ligation of inhibitory receptors, killing of the target cells ensues [19]. KIR expression is genotype-dependent, and coordinated so that different subsets of NK cells express KIRs detecting different HLA class I alleles, enabling the accurate detection of lacking HLA class I molecules. NK cells effectively kill virally infected cells and tumour cells, which express an altered HLA class I repertoire ("missing self") in order to escape immune recognition by Tc cells. The killing of virally infected cells by NK cells normally precedes the specific $CD8^+$ T cell response [10]. NK cells are capable of killing multiple cancer cell types in vitro, but their effectiveness against solid tumours remains poorly documented, possibly because tumours promote expansion of regulatory T cells, which inhibit NK cell function [20, 21].

In addition to their cytolytic activity, NK cells may play a major role in polarising T cell responses towards a Th1 response, as $CD56^{bright}$ NK cells are competent producers of IFN-γ, TNF-α and GM-CSF [22]. In the steady state, this subset comprises only 5-10% of human blood NK cells, but 75-95% of lymph node NK cells [23, 24], indicating that lymph node NK cells are important in early T cell polarisation in vivo, which has also been shown in the mouse [25]. NK cells express TLR3, TLR5 and TLR9, and thus stimulation of TLR3 with double-stranded RNA, TLR5 with bacterial flagellin, or TLR9 with CpG DNA are ways of inducing cytokine production in NK cells [26, 27], although the main activators of NK cell functions besides tumour cells appear to be DC.

Interactions between dendritic cells and natural killer cells

NK cells normally only kill autologous cells in the case of malignant transformation or virus infection, but immature myeloid DC constitute an exception from this rule. Immature DC express sufficiently low levels of HLA class I molecules, in humans together with unknown ligands for the activating receptor NKp30, to make them a NK cell target [28, 29]. It has been speculated that NK cells in the lymph nodes and in inflamed tissues also serve a regulatory function, as they may kill immature DC, which could otherwise be matured by cytokines and impede appropriate termination of an immune response [13]. Maturation of DC by microbial products, inflammatory mediators or, interestingly, by culture with NK cells, increase their expression of HLA-E and protects them from NK cell lysis [30, 31]. NK cell lysis of autologous DC has been shown to be impaired in different patient groups, but the in vivo significance of this phenomenon remains to be established [32, 33]. In addition to the selective killing or maturation of DC by NK cells, DC (including pDC) activate NK cells in several ways. Mature DC stimulate NK cells to proliferate and increase their cytotoxic activity against tumour targets [29, 34]. DC also induce cytokine production by NK cells in vitro and in vivo, principally IFN-γ [35, 36], and it has been shown that NK cells and DC co-localise

to T cell areas in the lymph nodes, where they may amplify each others cytokine production and polarise T cell responses. The DC-NK bi-directional cross talk is dependent on cell-to-cell contact, DC-derived cytokines (IL-12, IL-15, IL-18, IFN-α/β) and NK cell-derived cytokines (IFN-γ, TNF-α and GM-CSF) (Figure 1.2).

The interaction between DC and NK cells taking place before an adaptive immune response is established has been shown to be crucial in murine cytomegalovirus infection [6] and in establishing anti-tumour $CD8^+$ responses [37]. FMS-like tyrosine kinase 3 (Flt3) is a receptor present on haematopoietic stem cells, and its ligand, Flt3-L, is a growth factor promoting the expansion of immune cells of both the myeloid and the lymphoid lineage. Flt3-L administered to mice increases the total amount of DC in all organs 5-20 fold and also expands NK cell populations [38]. In vivo, Flt3L administration has been used as a means to show DC involvement in oral tolerance induction [39], and has also been shown to protect against experimentally induced asthma [40], indicating that the NK-DC axis could play a regulatory role in the immune system. In the mouse, a cell type with both DC and NK cell characteristics named interferon-producing killer DC has recently been identified [41, 42], but the physiological relevance of this cell subset and whether it constitutes a developmental stage of DC or NK cells has not yet been established.

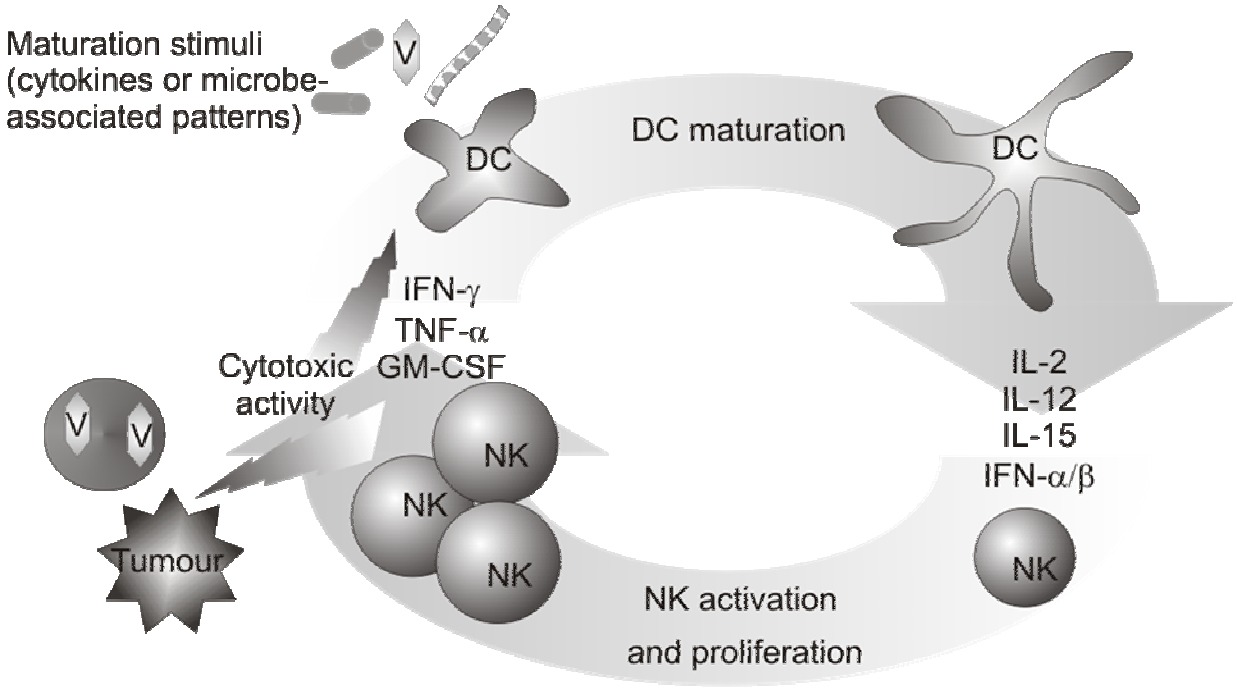

Figure 1.2: Bi-directional interactions between dendritic cells (DC) and natural killer (NK) cells. Mature and maturing DC secrete cytokines, which activates NK cells to proliferate, produce cytokines and increase their cytotoxic potential against virally infected cells and tumour cells. Activated NK cells in turn can either mature DC or kill immature DC via cell-to-cell contact and cytokine secretion.

Gut-associated lymphoid tissue

The largest mass of lymphoid tissue of the body is associated with the intestinal system, and these organised lymphoid structures in the intestine are termed the gut-associated lymphoid tissue (GALT). The mesenteric lymph nodes (MLN) are draining the intestinal lymphatics, but also in the intestinal wall itself, lymphoid structures exist (Figure 1.3). The most organised lymphoid tissues are the Peyer's patches (PP), which are clusters of multiple lymphoid follicles beneath the epithelium in the small intestine. The epithelium overlaying PP transforms into microfold (M) cells, which in contrast to normal epithelial cells, transport intact antigen over the epithelial layer [43]. Both in the small intestine and in the colon, similar but smaller structures, termed isolated lymphoid follicles, are present [44]. Below the epithelial layer, connective tissue and muscle form the lamina propria (LP), and within this supportive membrane, multiple leukocytes are found. Finally, disseminated lymphocytes are intercalated between the epithelial cells [43]. These intra-epithelial lymphocytes are primarily NK cells and T cells expressing either the common TCR composed of an α- and a β-chain, or the $\gamma\delta$ TCR [14, 43].

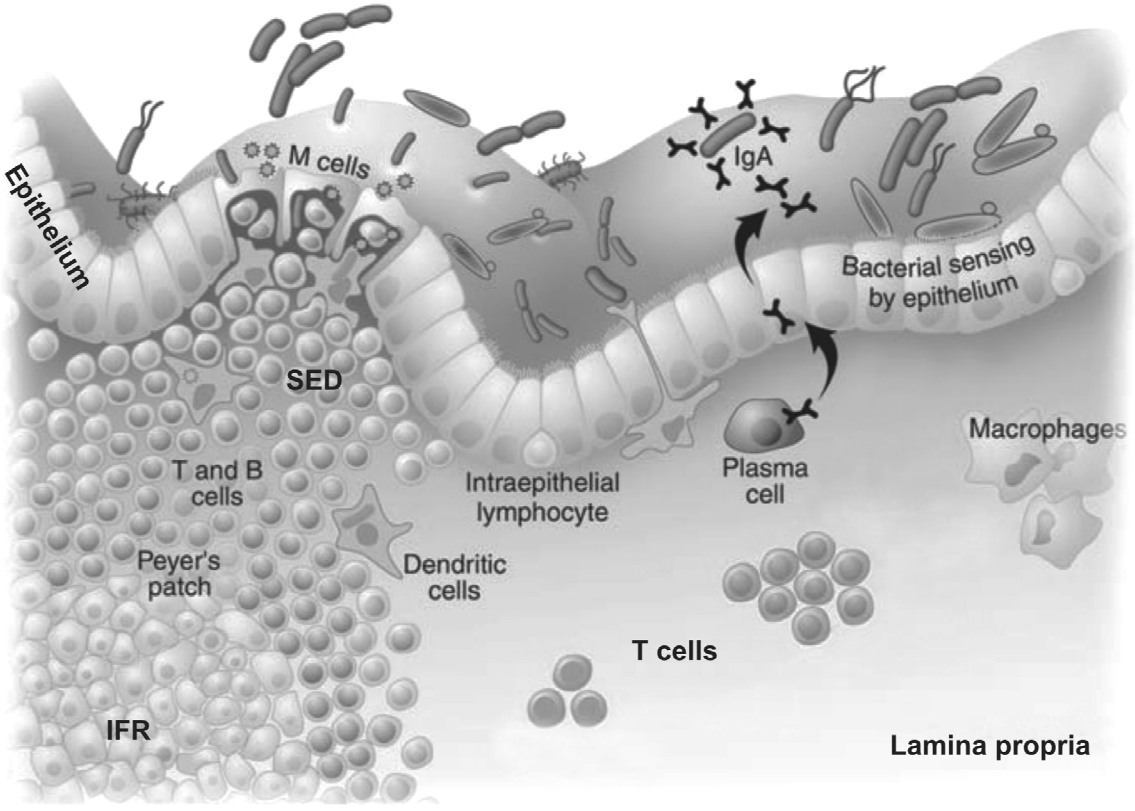

Figure 1.3: Cross-sectional representation of the gut-associated lymphoid tissues comprising Peyer's patches with overlying M cells, the lamina propria populated by T cells, dendritic cells, macrophages and IgA-producing plasma B cells, and the intra-epithelial lymphocyte compartment. SED: subepithelial dome region; IFR: Interfollicular region. Adapted from MacDonald & Monteleone [45].

One of the major roles of the GALT is to establish tolerance to ingested antigens and to the gut microbiota, while maintaining the ability to mount an active immune response in case of infection. So-called oral tolerance to low doses of fed antigens is antigen-specific and probably maintained by regulatory T cells [4]. Antigen enters the body via M cells overlying PP, or the LP (villous M cells) [46], where it is taken up by APC and presented to T cells or directly to B cells [47]. To keep the load of antigen to a minimum, large amounts of secretory IgA are produced by GALT [48]. IgA is an "antigen-excluding" antibody, which binds to antigens in the intestinal lumen or within epithelial cells and prevents antigen from accessing the body [49]. It is therefore also an anti-inflammatory antibody subclass, as opsonisation and involvement of phagocytic cells are not needed for antigen clearance. B and T cells primed in the gut migrate to MLN before they enter the bloodstream to later return to the GALT or other mucosa-associated lymphoid tissues [43].

Dendritic cells in the gastro-intestinal environment

The GALT is rich in DC present both in PP, in the MLN and in the LP. GALT DC have only been well characterised in the mouse, which is also the focus of work in GALT DC in this thesis, and therefore only mouse DC subsets will be described here. Murine DC are defined by their high expression of CD11c, but they differ in expression of CD4, CD8 and CD11b, with $CD11b^+$ cells being the subset resembling human myeloid DC the most. The $CD8^+$ DC subset of the mouse has been termed "lymphoid" DC, as it has diverse roles, including antigen cross-presentation to $CD8^+$ T cells. However, based on TLR-ligand responsiveness, not the $CD8^+$ subset but a $CD11c^{intermediate}$ DC subset seems to be the mouse equivalent of the human pDC [10]. The number of DC belonging to the main subsets differs between intestinal sites and from the DC composition of the spleen, the main difference being that $CD4^+$ DC are absent from the GALT [43]. All GALT DC are immature in the unstimulated state, although they may express slightly higher levels of co-stimulatory molecules than spleen DC (Table 1.3).

Table 1.3: DC subsets in mouse lymphoid organs. [43, 50-53]. ND: Not determinated.

Tissue	**Major DC subsets (all are $CD11c^+$)**			**$CD11c^+CD103^+$ DC (the majority are $CD11b^-$ and $CD8\alpha^{+/-}$)**	**Expression of the maturation markers MHCII / CD40 / CD80 / CD86 relative to spleen DC (reference)**
	$CD11b^+$ $CD8\alpha^-$	**$CD11b^-$ $CD8\alpha^+$**	**$CD11b^-$ $CD8\alpha^-$**		
Spleen	60-75%	20-30%	< 10%	30%	-
Mesenteric lymph nodes	30-40%	30-35%	30-40%	40%	ND/equal/++/equal [52]
Peyer's patches	30-40%	30-35%	30-35%	ND	equal/+/-/- [51], ND/++/+/equal [52]
Small intestine lamina propria	50-60%	15-20%	15-20%	60-70%	+/+/+/+ [51], ND/-/+/- [52]

In PP, $CD11b^+$ DC reside in the subepithelial dome region, ready to interact with antigen entering via M cells, whereas $CD8^+$ DC are present in the interfollicular region [54], and double negative DC are found at both sites. DC in the subepithelial dome region of the PP selectively express CCR1 and CCR6 and migrate towards the interfollicular region or towards the follicle-associated epithelium upon microbial stimulation [55], whereas all PP DC express CCR7 [54]. It has been suggested that $CCR6^+$ DC are recruited from the blood and remain in the subepithelial dome region until they encounter antigen [56]. $CD11b^+$ DC from PP mainly secrete IL-10 upon stimulation, whereas $CD8^+$ DC and double negative DC secrete IL-12p70 and induce Th1 responses [56]. Total PP DC predominantly secrete IL-10 and TGF-β, which may favour Th2/Th3 responses in the absence of infection. PP DC are also able to secrete IL-6 and induce IgA class-switching in B-cells [57]. PP DC migrate to MLN, and PP DC are necessary for controlling bacterial and parasite infections [55, 58].

LP DC send spherical protrusions through tight junctions between epithelial cells to sample the luminal content when bacteria have been detected by TLRs on the epithelial cells [59, 60]. They may also detect antigen taken up through isolated villous M cells found scattered in the epithelium. It has been suggested that resident intestinal DC have a tolerogenic phenotype, inducing regulatory T cells in the absence of pathogens [56]. It is plausible that there is a division of labour between subsets of DC, enabling certain subsets to respond to infection. However, recent data suggests that intestinal DC do not have intrinsic regulatory properties, but become less stimulatory upon exposure to micro-environment factors, e.g. thymic stromal lymphopoietin (TSLP) produced by epithelial cells [61]. Unless suppression is overcome upon pathogen exposure, this constantly expressed tissue factor may necessitate recruitment of unexposed DC from the blood to respond to infection.

By removing the MLN in rats it has been possible to study the DC contents of the lymph directed to the MLN. DC constitutively traffic to the MLN also in the absence of infection, and they have a life span of approximately 24 hours. Many DC of the MLN express the integrin subunits α_L and β_7 in a manner comparable to LP DC, so it is likely that MLN DC are LP-derived, although PP DC are also present in the MLN [52]. The receptor for the chemokines CCL19 and CCL21, CCR7, is required for the steady-state migration of LP DC, and is expressed on relatively immature DC [52], in contrast to DC migrating to other peripheral lymph nodes, which are generally mature. The function of this continuous exchange of information between LP and MLN has not been determined, but it is possible that DC carry antigens from apoptotic epithelial cells to maintain tolerance [52]. Certain MLN DC are essential in maintenance of oral tolerance but it is not known whether these DC originate in the LP, PP or elsewhere

[56]. Interestingly, CD103+ DC dominate in LP and are also present in MLN, and this subtype of DC is indispensable for generation of CD8+ T cell effectors and regulatory T cells homing to the gut [50, 53].

Commensal intestinal bacteria and the immune system

The immune response to commensal intestinal bacteria

The gastro-intestinal system of humans harbours 10^{14} bacteria, the so-called commensal bacteria. Low bacterial counts are found in the stomach (10-1000/ml content), increasing in the small intestine and rising to 10^{12}/ml in the colon (Figure 1.4). More than 500 species are present in the colon with predominant genera being *Bacteroides* and *Clostridium* [62, 63]. The main site of interaction between the intestinal flora and the GALT in both humans and rodents is believed to be the small intestine, as PP and most of the IgA-producing plasma cells are found here [64] together with the epithelium-spanning DC [60]. In addition, the colon is covered in a viscous mucus layer, beginning in the caecum and distally increasing in width, largely excluding bacteria from direct contact with the epithelium [65].

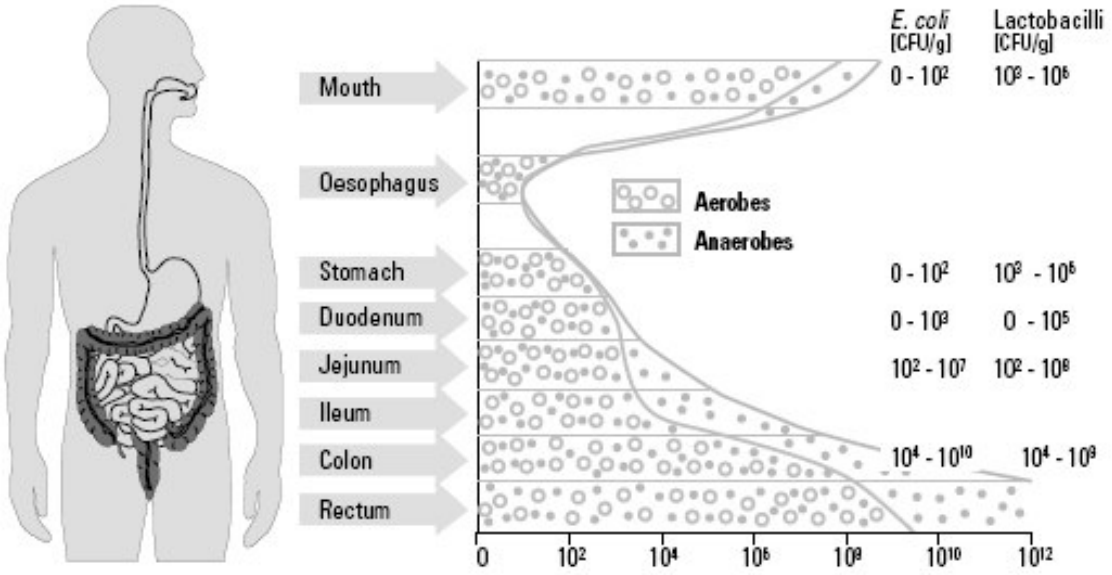

Figure 1.4: Numbers of commensal bacteria in different parts of the gastro-intestinal system. CFU: colony-forming units. From Schulze et al. [66].

Commensal bacteria transported as DC cargo can be found in MLN but, under normal conditions, not in the more distant lymphoid organs [67]. Data from studies in gnotobiotic, i.e. non-colonised, rodents and in rodents with a controlled microbiota suggest that the resident microbiota enter in a complex relationship with their animal host, benefiting health via interactions with both the intestinal and the systemic immune system. If the immune system is not stimulated by gut bacteria after birth it

does not develop completely, comprises fewer T cells (including Tregs) [68, 69], and generates weaker antibody responses [70]. The microbial stimulation skews the immune system away from the neonatally dominating Th2 response towards a balanced Th1 immune profile [71]. Remarkably, the number of DC in the MLN is reduced in germ-free mice compared to mice with a normal intestinal microbiota, while maturation status and function of the DC are unaltered [72].

Protein antigens derived from commensal microorganisms have been shown to elicit only IgA responses, and not IgG responses in normal mice [73]. This may indicate that the systemic immune system remains ignorant of the intestinal flora, at least concerning protein antigens [67]. However, a "cocktail" of enteroantigen was shown to elicit in vitro T cell responses when regulatory T cells were removed, perhaps indicating that the systemic immune system reacts towards intestinal bacteria found outside the tolerogenic gut environment [74]. In addition, it is believed that carbohydrate structures of the commensal flora play an important role in shaping the systemic immune system [69]. In consequence, the "naïvety" of the systemic immune system towards the microbiota of the gut and other mucosal sites deserves further investigation.

Lactic acid bacteria and probiotics

Lactic acid bacteria (LAB) is a group of bacteria characterised by their ability to produce lactic acid and comprise bacteria belonging to several genera. Most species of LAB belong to the genera *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc* or *Lactobacillus*. LAB are predominantly non-pathogenic, Gram-positive, catalase-negative, non-sporeforming, facultative anaerobes and obligate fermentative. Traditionally, these bacteria have been applied in food fermentations due to their lactic acid production contributing acidity and thereby prolonged shelf life to the foodstuff, and to the pleasant flavour of other metabolites [75]. Bifidobacteria are not considered true lactic acid bacteria, as their main metabolite is acetic acid, but as they share most properties with and are used in similar products as LAB they are also commonly referred to as LAB.

Some strains of lactobacilli and bifidobacteria are acid and bile tolerant and can be isolated from the mammalian gastro-intestinal system. In the stomach and in the upper part of the small intestine, lactobacilli outnumber other bacteria (Figure 1.4), whereas bifidobacteria are mainly present in the anaerobic colon, as the dominant genus in breast-fed infants and in adulthood co-existing with many other bacterial genera [76]. In consequence, foods containing viable LAB such as yoghurt have gained a reputation of benefiting intestinal well-being, and LAB strains with assumed health effects have been termed 'probiotic' (pro-life, as opposed to antibiotic). 'Probiotic' can also be assigned to other microorganisms, for instance certain strains of *Escherichia coli* and *Saccharomyces boulardii*, but the

majority of commercial probiotics are LAB [64]. The beneficial effect of probiotics on intestinal health can be assigned to their ability to competitively exclude pathogenic bacteria, to degrade indigestible food components (i.e. fibre or lactose in lactose-intolerant individuals) to valuable nutrients, and finally to support epithelial cell growth and intestinal wall impermeability [77]. The definition of probiotics currently employed by the World Health Organization is: 'Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' [78].

Lactic acid bacteria as immunomodulators

In addition to the overall dependency of the immune system on gut colonisation for development, it has been shown that individual strains of commensal bacteria, including strains of LAB, possess varying immunomodulatory properties and may finely polarise the immune response. In vitro, LAB mainly exert their effects on APC such as DC, Mo and macrophages, without necessarily being the source of antigen. Cytokines secreted by and surface molecules on APC induced by LAB-stimulation are, together with the type of antigen encountered, determinants of an ensuing T cell response [79]. Comparisons of stimulation of Mo and macrophages with LAB as opposed to Gram-negative bacteria show that LAB preferentially induce IL-12 production, whereas Gram-negative bacteria such as *E. coli* mainly induce IL-10 [80-82].

LAB stimulation of DC, on the other hand yields a more complicated picture, as different strains of the same genus or even species of LAB can induce different amounts of IL-10 and IL-12 [83, 84]. In addition to these Th1-Th2/Treg polarising cytokines, LAB stimulation frequently leads to TNF-α and IL-6 production in Mo and DC [84, 85]. Concomitantly with induction of cytokine production, different LAB induce different levels of maturation in DC. Overall, LAB induce 'semi-mature DC', which express lower levels of CD40, CD80 and CD86 than DC matured by pathogenic Gram-positive bacteria and Gram-negative bacteria in general [84, 86], and induction of maturation markers correlate with their capacity to induce IL-12 and TNF-α. Among gut-derived LAB, *Lactobacillus*-dependent induction of surface markers varies significantly with the strain, whereas most bifidobacteria induce low levels of maturation [84].

Immunomodulatory components of lactic acid bacteria

Consensus has not been reached regarding whether bacteria have to survive passage of the gastrointestinal tract to be termed probiotic. However, a picture is emerging where live bacteria are required in the gut to compete with pathogens and to metabolise lactose and fibres (although also dead bacteria may contribute with carbohydrate-degrading enzymes) [87], whereas effects of LAB on the immune system can be observed with both viable and dead bacteria [84, 88]. For some

immunomodulatory effects cell integrity is required [89], but in other cases bacterial components are stimulatory by themselves. Candidate active molecules of LAB are DNA [90, 91] and cell wall structures [92, 93]. CpG DNA is a ligand of TLR9, and cell wall peptidoglycan (PG), lipoteichoic acid (LTA) and lipopeptides are recognised by TLR2, some in conjunction with TLR1 or TLR6 [94]. In contrast, Gram-negative bacteria, which are also abundant in the gut, are detected through interaction of their lipopolysaccharide (LPS, endotoxin) by TLR4 and to a minor extent TLR2 (Table 1.1).

Microbial ligation of TLRs results in translocation of nuclear factor κB (NFκB) to the nucleus with secretion of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) as a consequence. LAB-induced IL-12 production is also likely to be a product of TLR-stimulation, as expression of the subunit IL-12p40 in DC in the LP of the small intestine requires binding of NFκB [95], but also intracellular Nucleotide-binding oligomerisation domain (Nod) receptors could be involved, as they recognise PG [96]. Regarding the suppressive cytokine IL-10, it is induced in DC when microbes are recognised by TLRs or via C-type lectin receptors such as DC-SIGN, and when CD40 is ligated by the CD40 ligand on T cells [79, 97]. In studies from our lab, bacterial strains, which presumably possess TLR ligands of the same type, induce intriguingly different DC maturation and cytokine inductions patterns [83, 84]. Both for these LAB strains and for pathogenic microorganisms the signalling pathways downstream of the TLRs permitting such variety remain poorly understood.

T cell polarising effects of lactic acid bacteria

DC are considered the most important type of antigen presenting cell, as it is the only cell type capable of presenting antigen to naïve T cells. The polarising capacities of LAB-stimulated DC, producing high amounts of IL-10, IL-12 and other polarising cytokines [84], are therefore an area of interest. In vitro, the T cell polarising properties of DC stimulated with LAB has mainly been studied in human cells, which require allogeneic or non-specific stimulation to mimic an antigen-specific response and to obtain production of cytokines characteristic for Th1-, Th2-, or Treg cells. DC matured by a *L. rhamnosus* strain combined with inflammatory cytokines do not change the levels of IL-4, IFN-γ or IL-10 secreted by naïve T cells stimulated by DC and *Staphylococcus aureus* enterotoxin B [98]. Strains of *L. reuteri*, *L. casei* and *L. plantarum* employed under the same conditions do not alter the Th1/Th2 balance either, but *L. reuteri* and *L. casei* interestingly induce IL-10 production in T cells and the T cells exert a modest regulatory activity on bystander T cell proliferation [99]. In contrast, DC matured by Gram-negative bacteria, including commensal *E. coli* strains induce Th1-responses [89]. Th1 polarising properties have also been reported for allogeneic DC matured by *L. reuteri*, *L. gasseri* and *L. johnsonii* [85], indicating that the

polarising potential of LAB-matured DC is strain-dependent, but probably also depends on the accessory T cell stimuli employed. Strong evidence for Th1-skewing potential of LAB comes from a study wherein DC were generated from allergic donors. These DC induce IL-4 and IL-5 in autologous T cells in the presence of the allergen, but in the presence of *L. plantarum,* production of these Th2-cytokines was reduced at the expense of increased IFN-γ production [100]. Interestingly, native LTA of this *L. plantarum* strain induce IL-12 and IFN-γ via TLR2 in PBMC, while a modified LTA structure preferentially induces IL-10 [92]. Regarding potential polarisation towards the ThIL-17 phenotype, Smits et al. [89] have demonstrated that strains of *B. adolescentis*, *L. plantarum* and *Enterococcus faecalis* do not induce production of the ThIL-17-sustaining cytokine IL-23 in DC.

Table 1.4: Double-blinded placebo- or dummy-controlled trials for prevention of recurrence in inflammatory bowel disease.

Aim of therapy	Probiotic strains	Dose and intervention period	Outcome in probiotics group (reference)
Prevention of relapse in ulcerative colitis	*E. coli* Nissle 1917	$5{\cdot}10^{10}$ bacteria daily for 12 months	**As effective** in prevention of relapse as the standard drug mesalazine [101]
	E. coli Nissle 1917	$5\text{-}50{\cdot}10^{9}$ bacteria daily for 12 months	**As effective** in prevention of relapse as mesalazine [102]
Prevention of pouchitis onset or relapse	Mixture of *L. acidophilus, L. delbrueckii* ssp. *bulgaricus, L. casei, L. plantarum B. breve, B. longum, B. infantis, and Streptococcus salivarius* ssp. *thermophilus* (VSL#3)	$3{\cdot}10^{11}$ bacteria daily for 12 months	**Effective** in preventing relapse [103]
	VSL#3	$9{\cdot}10^{11}$ bacteria daily for 12 months	**Effective** in preventing pouchitis onset [104]
	VSL#3	$5{\cdot}10^{11}$ bacteria daily for 9 months	**Effective** in preventing relapse [105]
	L. rhamnosus GG	10^{10} twice daily for 3 months	**Ineffective** in preventing relapse [106]
Prevention of relapse in Crohn's disease	*L. rhamnosus* GG	$1.2{\cdot}10^{10}$ bacteria daily for 12 months	**Ineffective** in preventing relapse [107]
	L. rhamnosus GG	10^{10} bacteria twice a day for up to 2 years	**Ineffective** in preventing relapse [108]
	L. johnsonii LA1	$2{\cdot}10^{9}$ bacteria twice a day for 6 months	**Ineffective** in preventing relapse [109]

Anti-allergy and anti-inflammation effects of lactic acid bacteria

Although in vitro studies of T cell polarisation are scarce it is believed that T cell polarisation by LAB is important in vivo. Consumption of certain LAB has been shown to alleviate allergy in mice [110], and it is assumed that LAB skew the allergic Th2 response to harmless antigens to a Th1 response via the induction of IL-12 in APC, likely DC. It may also be that LAB induce regulatory T cells capable of

suppressing the allergic Th2 response. Such a regulatory function is believed to dominate in several mouse models of inflammatory bowel diseases (IBD, diseases occurring when the GALT immune system reacts inappropriately to the commensal microbiota), wherein administration of LAB alleviates symptoms [92, 111]. The VSL#3 mixture of LAB strains (for composition see Table 1.4) reduces diabetes incidence in non-obese diabetic mice and increases IL-10 production in pancreas and PP cells [112], which may be due to enhanced Treg function. Some studies suggest additional roles for LAB Th1 immunomodulation in cancer prevention [113, 114], and Th1 polarisation by the gut microbiota may also play a role in inhibiting Th-IL-17 polarisation leading to autoimmunity, although this possibility has not yet been addressed experimentally.

Table 1.5: Double-blinded placebo-controlled trials of probiotics for prevention or alleviation of atopic dermatitis (AD).

Age group	Probiotic strains	Dose and intervention period	Outcome in probiotics group (reference)
Newborns with allergic mothers, fathers, or close relatives	Mixture of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. Breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii*	Mothers: > 10^{10} bacteria/day for 2-4 week pre-delivery. Infants: the same probiotics for 6 months	**Reduction** in eczema and atopic eczema after 2 years, [115]
	L. rhamnosus GG	Mothers: $2{\cdot}10^{10}$ bacteria/day for 2-4 week pre-delivery and during breastfeeding for up to 6 months. Formula-fed infants: the same probiotics for 6 months	**Reduction** in AD incidence [116]
	L. acidophilus LAVRI-A1	$3{\cdot}10^{9}$ bacteria/day for 6 months	**No reduction** of AD incidence, increased risk of allergen sensitisation [117]
Infants < 18 months old with AD	*L. rhamnosus* GG or *L. rhamnosus* NP-Lrh	$5{\cdot}10^{9}$ bacteria/100 ml formula ad libitum for 3 months	**No alleviation** of AD [118]
	L. rhamnosus GG or *B. lactis* BB-12	$3{\cdot}10^{8}$ – 10^{9} bacteria/g formula ad libitum for 2 months	**Alleviation** of AD in both probiotics groups [119]
	L. rhamnosus GG or mixture of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. Breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii*	$5{\cdot}10^{9}$ *L. rhamnosus* GG or > 10^{10} bacteria of the mixture twice daily for 4 weeks	**Alleviation** of AD **only** in IgE-sensitized children [120]
	L. rhamnosus GG	$5{\cdot}10^{9}$ bacteria/100 ml formula ad libitum for 1 month	**Alleviation** of AD [121]
	L. fermentum VRI-033	10^{9} bacteria twice daily for 8 weeks	**Alleviation** of AD in the probiotics group [122]
Children 1-13 years old with AD	*Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 12246	10^{10} bacteria of each strain daily for 6 weeks	**Alleviation** of AD [123]

In humans, LAB and other probiotics have proven beneficial in a limited number of conditions including antibiotics-induced and rotavirus diarrhoea [124], childhood allergy and recurrence of

certain types of IBD (refs. in Table 1.4 and 1.5). Atopic dermatitis (AD) in infants and IBD are the diseases in which most controlled intervention studies with probiotic bacteria have been conducted. Moreover, these diseases are immune-mediated, and there is therefore reason to believe that immunomodulation by probiotics may have an impact. Considering only these double-blinded placebo- or dummy-controlled trials, there is an indication that administration of the LAB strain mixture VSL#3 or the probiotic *E. coli* strain Nissle 1917 can prevent recurrence of ulcerative colitis and pouchitis, but LAB do not prevent recurrence in Crohn's disease (Table 1.4). Some probiotics can reduce the prevalence or the severity of atopic dermatitis in infants, especially when administered to the mother pre- and post-delivery (Table 1.5). Several studies have, however, failed to show an effect of probiotic supplementation. From the limited number of studies it is difficult to judge whether the dose, the strain of probiotic bacteria, or both are critical, but the key to successful treatment of aberrant immune responses may be careful consideration of the T cell polarising capacities of the probiotic strains, and administration in very high numbers.

Lactic acid bacteria inducing effector functions in natural killer cells

It should be taken into account that cell types other than APC and T cells play a role in T cell polarisation. NK cells are considered to play a key role in the induction of Th1 responses as early producers of IFN-γ in lymph nodes [25]. It is not known whether NK cells secreting IFN-γ after interaction with DC participate in the systemic Th1-polarisation observed for certain LAB. However, as LAB have a profound modulatory effect on DC and because DC in turn are potent activators of NK cells, it is reasonable to expect that DC, which have encountered LAB in the GALT and undergo maturation can stimulate intra-epithelial NK cells or NK cells in the MLN. Similarly, LAB-stimulated Mo produce IL-12 and induce IFN-γ production in NK cells [80, 125], which may also promote Th1-polarisation, if this interaction takes place in vivo.

A number of probiotic LAB - both IL-12-inducing and non-IL-12-inducing strains - have been tested for their ability to increase in vivo NK cell activity as a measure of innate immune activity after promising findings in animal studies [126]. Intervention studies have been conducted in healthy volunteers, of which the cytolytic potential of PBMC against standard tumour target cell lines has been measured at several stages of the intervention. Evidence of an increase in NK cell activity after probiotic supplementation has been found in elderly individuals [125, 127, 128] and in habitual smokers [129]. Often, the increment in NK cell cytolytic activity is lost when probiotic supplementation is terminated [127, 128], reflecting that probiotic bacteria rarely colonise the host permanently [130]. DC, and not Mo, are likely to be the cell type responsible for the increase in NK cell activity, as DC present in PP and LP have access to the commensal microbiota and continuously migrate to MLN [60]. In mice, increased cytolytic

potential of NK cells after ingesting probiotic bacteria has been correlated to a reduction in tumour incidence [114]. Investigators have attempted to assign the increase in NK cytolytic activity after probiotic supplementation to either an increase in NK cell numbers or in per-cell cytotoxicity, but both phenomena seem to occur [114, 128]. In addition to a direct effect of NK cells on tumours, NK cell activation may promote Th1 polarisation and thereby a cytotoxic T cell anti-tumour response.

Thesis outline

Gut bacteria, including lactic acid bacteria, are strong inducers of maturation in DC. As mature DC engage in cross-talk with NK cells and T cells, gut bacteria are likely to have an effect on these effector cells. This thesis describes the in vitro immunomodulatory effect of gut-derived bacterial strains on different immune cells, in particular human and murine DC, and the resultant effect of DC-stimulation of NK and T cell effector functions. These studies were undertaken to assess whether DC cytokine secretion and expression of maturation surface markers in response to gut-derived bacteria are indicative of the potential of DC to prime NK and T cell responses, and to evaluate the validity of DC generated in vitro as a model of blood and intestinal DC. The cell types studied in the different chapters are shown in Figure 1.5.

In Chapter 2, we describe how the differential effect of LAB strains on human monocyte-derived DC polarisation caused induction of different effector functions in NK cells cultured with LAB-matured DC. In particular, IL-12 secreted by DC governed IFN-γ production by NK cells. This finding is in Chapter 3 shown to apply also to freshly isolated blood DC and monocytes. In addition, we investigated the T cell polarising potential of monocyte-derived DC, blood DC, and monocytes stimulated with gut-derived bacterial strains. In Chapter 4, the response to gut-derived bacteria of DC isolated from different mouse tissues including MLN and PP is characterised, and a role for NK cells in the response to gut bacteria is suggested. Chapter 2 and Chapter 3 have been accepted for publication in International Immunology and FEMS Immunology and Medical Microbiology, respectively.

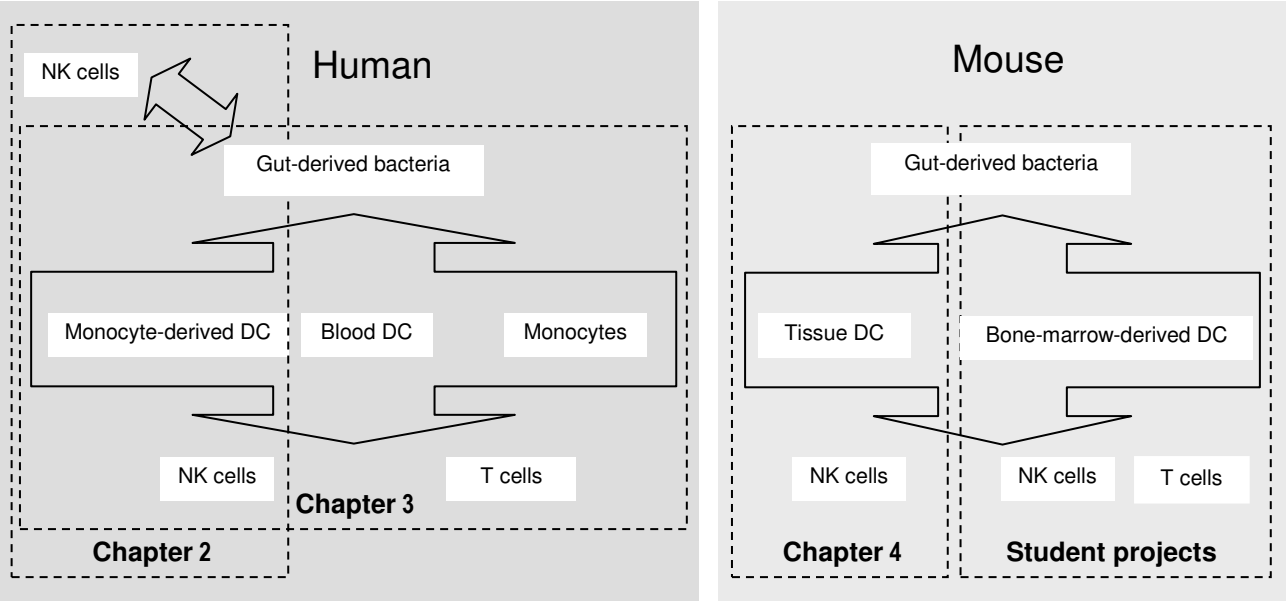

Figure 1.5: Overview of the cell types used to study the immunomodulatory properties of gut-derived bacteria in each chapter of the thesis.

2. Distinct strains of gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated natural killer cell responses

In collaboration with Louise H. Zeuthen, Hanne R. Christensen, Barbara Morandi, Hanne Frøkiær[1] and Guido Ferlazzo[1].

Abstract

Lactic acid bacteria (LAB) are abundant in the gastro-intestinal tract where they continuously regulate the immune system. Natural killer (NK) cells are potently activated by dendritic cells (DC) matured by inflammatory stimuli, and NK cells are present in the gut epithelium and in mesenteric lymph nodes, but it is not known how NK-DC interactions are affected by the predominantly non-pathogenic LAB. We demonstrate that human DC exposed to different strains of gut-derived LAB consistently induce proliferation, cytotoxicity and activation markers in autologous NK cells. On the contrary, individual strains of LAB differ greatly in their ability to induce DC-dependent IFN-γ production by NK cells. This suggests that DC stimulated by gut LAB may expand the pool of NK cells and increase their cytotoxic potential. Specific LAB, inducing high levels of IL-12 in DC, may promote amplification of a type 1 response via potent stimulation of IFN-γ production in NK cells. Combining IFN-γ-inducing and non-inducing LAB strains completely abrogates DC-mediated IFN-γ production by NK cells, and therefore LAB strains modulating IFN-γ production in NK cells may be important regulators of the immune response.

Introduction

Mechanisms linking innate immune function with adaptive immunity are gaining interest, and much attention has been given to the interactions between myeloid dendritic cells (DC) and natural killer (NK) cells. NK cells are capable of initiating cytokine production in the early phase of an immune response and kill cells expressing an altered MHC-repertoire and specific stress-induced molecules [19]. The bi-directional cross-talk between NK cells and DC includes induction of IFN-γ production [34, 131], activation markers [30], increased proliferation [29, 132] and cytotoxic activity [27, 34] in NK cells by mature DC (mDC), as well as maturation of immature DC (iDC) by NK cells [30, 131, 132]. It has also been proposed that immature DC that are not successfully matured upon contact to NK cells are killed by activated NK cells [133], possibly permitting survival only of DC that have encountered relevant antigen. Maturation of

[1] Hanne Frøkiær and Guido Ferlazzo contributed equally to the work presented in Chapter 2.

DC by inflammatory mediators such as TNF-α [29, 34] or TLR ligands [27, 131] has been widely used in the study of DC-NK interactions. In addition, maturation by intact pathogens confers NK cell-stimulatory activity to DC, as has been shown for *Escherichia coli*, *Mycobacterium bovis* BCG [134] and *Helicobacter pylori* [135].

Interactions between NK cells and DC are likely to occur in the gut-associated lymphoid tissue, where NK cells reside among intra-epithelial lymphocytes [14], or in the mesenteric lymph nodes through which gut DC continuously circulate [136]. The interaction between antigen-loaded mature DC and NK cells in lymph nodes has been described to result in prompt IFN-γ production, and to be necessary for Th1 polarisation of subsequent adaptive responses [25, 137], and this may also apply to the gut-associated immune system.

DC are important gate-keepers in the intestine. They reside in Peyer's patches, capturing antigen shuttled through M-cells [45], and in the lamina propria where they sample antigen that cross the epithelial barrier or send dendrites through tight junctions of the epithelium to sense antigen in the intestinal lumen [60]. Therefore, these cells are considered important in the diverse responses to pathogens versus harmless food antigens and commensal gut microorganisms elicited in the healthy intestine. Accumulating evidence shows that commensal bacteria play a role in educating immune cells of the gut, including DC, to induce appropriate responses to intestinal antigens [45].

Lactic acid bacteria (LAB) comprise several genera of which lactobacilli, bifidobacteria and enterococci are abundantly present in the intestinal tract; especially in the lower small intestine and colon [64]. LAB are widely used in fermented foods, wherein they are termed "probiotic" when considered beneficial to health [75]. One potential health-promoting property is modulation of the immune system. It has been previously demonstrated that different LAB strains induce variable levels of co-stimulatory molecules and cytokine release in murine and human DC, and that the effect of combinations of strains cannot be predicted from the properties of individual strains [83, 84]. Certain strains of *Lactobacillus* subspecies added during DC maturation have been shown to lead to DC that induce Tregs, whereas other strains do not [99]. DC matured by three specific *Lactobacillus* strains induce IFN-γ production in T cells [85], while Pochard et al. [100] observed that DC matured by a *L. plantarum* strain were able to skew T cells from allergic patients away from a Th2 cytokine response ex vivo. Thus, distinct LAB possess the ability to polarise DC towards Th1- or Treg-inducing phenotypes, and this may be the reason for beneficial effects observed following LAB administration in patients suffering from infectious diarrhoea and atopic diseases [130]. Nevertheless, mechanistic evidence for these effects is lacking.

An enhancement of NK cell activity after LAB consumption has been observed in a number of human intervention studies in healthy adults [138], elderly [127, 128, 139] and smokers [129]. When mice are fed LAB concomitantly with mutagen injections, increased NK cytolytic activity correlate with a decrease in tumour incidence and the positive effect of probiotic supplementation is absent in NK cell deficient beige mice [114]. A plausible explanation for the observed increase in NK cytolytic activity after intake of certain probiotic formulations is that NK cells interact directly with the non-pathogenic LAB or with DC stimulated by LAB.

The aim of this study was to characterise NK-DC interactions in response to gut-derived LAB having differential effects on DC. We report that DC matured by LAB consistently induce activation and promote proliferation and cytotoxicity in NK cells, and that strains of different LAB species differ importantly in their capacity to induce IFN-γ production in NK cells via DC.

Materials and methods

Cell isolation and culture

PBMC were obtained from buffy coats (Copenhagen University Hospital, Denmark and G. Gaslini Children's Hospital, Genoa, Italy) by centrifugation on Ficoll-Paque separation medium. Cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Cambrex Bio Science, East Rutherford, NJ) referred to as complete medium. $CD14^+$ cells were isolated from PBMC using anti-CD14 microbeads (Miltenyi, Bergisch Gladbach, Germany) and differentiated into immature DC (iDC) during 6 days of culture in a humidified 5% CO_2 atmosphere in complete medium supplemented with 20 ng/ml granulocyte-macrophage colony-stimulating factor (Biosource, Camarillo, CA) and 100 IU/ml IL-4 (Euroclone, Milan, Italy). Fresh medium containing cytokines was supplied on day 3 of culture. DC were incubated with UV-killed LAB (25 μg/10^6 DC/ml) in 24-well plates for 6 or 20 hours to yield mature DC (mDC). NK cells were negatively isolated from PBMC using the NK cell Isolation Kit II (Miltenyi). Isolated NK cells were consistently > 95% $CD3^-$ $CD56^+$ cells and devoid of monocytes. NK cells (10^5 cells/well) were cultured with autologous DC ($2{\cdot}10^4$ cells/well) with or without LAB (0.5 μg/$2{\cdot}10^4$ DC/0.2 ml) in round-bottom 96-well plates. IL-12 neutralisation antibody (2 μg/ml, R&D Systems, Minneapolis, MN) or IL-10 neutralisation antibody (2.5 μg/ml, BD Biosciences, Franklin Lakes, NJ) were added to cultures where indicated.

Bacterial strains

The strains of LAB used were: *L. reuteri* DSM12246, originally isolated from pig faeces [140], and *L. acidophilus* X37 and *B. bifidum* S13.1, isolated from a human intestinal biopsy and from a child's faeces, respectively. The latter two strains were isolated and typed at the Department of Food Science, University of Copenhagen, Denmark. LAB were grown in de Man, Rogosa and Sharpe medium (Merck, Darmstadt, Germany) until stationary growth phase, harvested by centrifugation at 2000 *g* for 15 min., washed twice with sterile PBS, resuspended in PBS and UV-irradiated for 15 min. in a thin film of liquid. Dry matter determinations were made in quadruplicates. LPS contamination of LAB suspensions was tested with the Pyrochrome kit (Ass. of Cape Cod, East Falmouth, MA). Endotoxin content was < 0.1 EU/ml for all LAB suspensions at the concentrations used in cell culture experiments. UV-killed LAB were stored at -80°C. To examine compounds released during bacterial growth, LAB were grown in antibiotics-free complete medium until stationary growth phase, centrifuged at 2000 *g* and the supernatant sterile filtered (0.2 μm). Filtered supernatants were added to DC at 10% of the culture volume.

Cytokine quantification

Cytokine concentrations in culture supernatants were determined using ELISA kits for IL-12p70 (R&D Systems) and IFN-γ (Biosource) according to the manufacturers' instructions. IL-10 was quantified by ELISA using a monoclonal antibody pair from BD Biosciences (clones JES319F1 and biotinylated JES3-12G8).

Cell surface marker analysis

Cells were incubated with human γ-immunoglobulin (human therapy grade) for 10 min. at room temperature to prevent binding of staining antibodies to Fc-receptors. Subsequently, cells were incubated for 30 min. at 4°C with unconjugated monoclonal antibodies against HLA-I (clone W6/32, kindly provided by Dr. M.C. Mingari, University of Genoa, Italy), HLA-DR (clone D12.1, kindly provided by Dr. G. Frumento, IST, Genoa, Italy) or CCR7 (BD Biosciences) followed by staining with appropriate FITC-labelled secondary antibodies, with FITC-labelled monoclonal antibodies against CD3 (BD Biosciences), CD80, CD83 (both Beckman Coulter, Fullerton, CA), PE-labelled monoclonal antibodies against CD25, HLA-DR (both BD Biosciences), CD69 (Ancell, Bayport, MN), NKp44 (Beckman Coulter), and/or with a PE-Cy5-labelled monoclonal antibody against CD56 (Beckman Coulter). Propidium iodide (Sigma, St. Louis, MO) was used to assess cell viability. Flow cytometric analysis of surface marker expression was performed on a FACSScan flow cytometer (BD Biosciences).

Proliferation assay

Cell proliferation during 4 days of co-culture of DC and NK cells was assessed by adding tritiated thymidine (Amersham Biosciences) to the cultures 20 hours prior to harvesting them onto glass filter mats using a cell harvester (AQS Manufacturing, Horsham, UK). Incorporation of tritiated thymidine was quantified in a scintillation counter (Packard, Meriden, CT). Results are given as counts per minute (cpm).

Cytotoxicity assay

NK cell cytotoxicity against the target cell line K562 was assessed in a 4 h Cr^{51} release assay. NK cells and Cr^{51}-labelled target cells were co-cultured in V-bottom 96-well plates at the indicated ratios for 4 hours at 37°C. Aliquots of labelled target cells were incubated without NK cells to quantify spontaneous Cr^{51}-release or lysed with 2 N HCl to assess total Cr^{51}-release. Cr^{51} released into the supernatant was quantified in a gamma counter (Beckman Coulter). The percentage of specifically lysed target cells was calculated as 100*(sample release – spontaneous release) / (total release – spontaneous release).

Statistical analysis

Statistical analysis (one-way analysis of variance with Tukey's post hoc test or two-way analysis of variance with Bonferroni post hoc test) was performed using the GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA). Differences were considered significant if $P < 0.05$.

Results

Gut-derived LAB induce species-dependent cytokine patterns in DC and DC-dependent IFN-γ release by NK cells

We have previously observed great differences in the amount of IL-12 produced by DC in response to different single and combined strains of LAB [83, 84]. Prior to investigating the effect of LAB-stimulated DC on NK cells, we assessed the cytokine pattern of DC cultured in the presence of single and combinations of three representative strains of gut-derived LAB. In accordance with our earlier results, monocyte-derived iDC cultured with UV-killed LAB for 20 h produced different amounts of IL-12 and IL-10 (Figure 2.1A). *L. acidophilus* induced the highest amount of IL-12 and slightly more IL-10 than the other bacteria. Adding a combination of *L. acidophilus* and *B. bifidum* or *L. reuteri* to iDC abrogated IL-12 production, whereas IL-10 production was additively increased.

The high ratio of IL-10 to IL-12 induced by *B. bifidum* and *L. reuteri* suggested that IL-10 is responsible for the inhibition of *L. acidophilus*-induced IL-12 production. When *L. acidophilus* and an IL-

12-inhibitory LAB strain were added together with an anti-IL-10 antibody, IL-12 production was indeed markedly increased (Figure 2.1B, left panel), but neutralising IL-10 also substantially increased IL-12 production induced by *L. acidophilus, B. bifidum* and *L. reuteri* added separately. The inhibitory effect of *B. bifidum* and *L. reuteri* on *L. acidophilus*-induced IL-12 production was maintained with increasing concentrations of anti-IL-10 antibody (Figure 2.1B, right panel).

When iDC and individual or combinations of LAB strains were added simultaneously to NK cells, the IFN-γ production by NK cells (Figure 2.1C) correlated with the amount of IL-12 induced in DC. As expected, *L. acidophilus*-induced IFN-γ production was significantly reduced when IL-12 was neutralised by an anti-IL-12 antibody, whereas addition of anti-IL-10 antibody slightly increased IFN-γ production. The IFN-γ production upon IL-10 neutralisation was, however, not increased to a degree matching the increase in DC IL-12 production. NK cells stimulated with different strains of LAB in the absence of DC did not produce IFN-γ. Large amounts of TNF-α were produced in the NK-DC co-cultures stimulated with LAB, especially with *L. acidophilus*, but TNF-α was mainly produced by DC and NK cells did not contribute significantly (not shown).

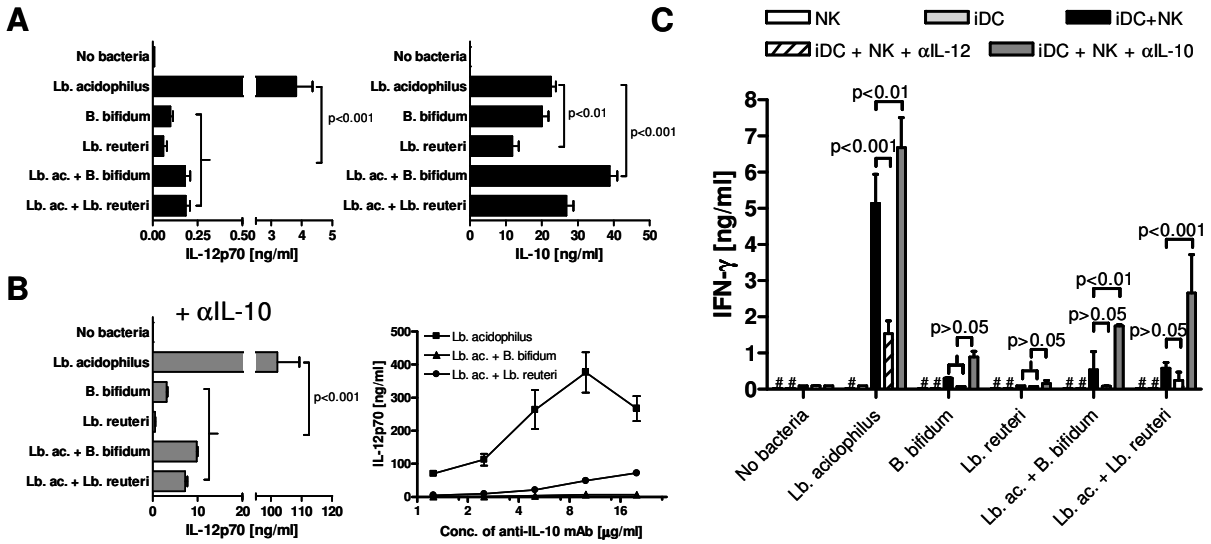

Figure 2.1: Distinct LAB induce different amounts of cytokines in DC and NK cells. (A) UV-killed LAB (*L. acidophilus*, *B. bifidum*, *L. reuteri*, or *L. acidophilus* combined with one of the other strains; 25 µg/10^6 DC/ml, 20 h) induced production of variable amounts of IL-12 and IL-10 in immature DC (iDC). (B) IL-12 production by iDC cultured with LAB increased for all stimuli upon addition of IL-10 neutralisation antibody, but the ability of *B. bifidum* and *L. reuteri* to reduce *L. acidophilus*-induced IL-12 production was maintained with increased concentrations of anti-IL-10 antibody. (C) UV-killed LAB (*L. acidophilus*, *B. bifidum*, *L. reuteri*, or *L. acidophilus* combined with one of the other strains; 0.5 µg/2·10^4 DC/0.2 ml, 48 h) induced IFN-γ production in NK cells (10^5/well) cultured with autologous iDC. IFN-γ production was reduced upon addition of anti-IL-12 antibody, but slightly increased when anti-IL-10 antibody was added. Cytokines in culture supernatants were

quantified by ELISA. Data are means and SD of duplicate cultures. "#" indicates: not detected. Data are representative of four experiments with cells from different donors.

To examine the nature of the bacterial stimuli evoking the differential cytokine production in DC and NK cells, we cultured LAB in cell culture medium until stationary growth phase and sterile filtered the supernatant. Adding 10% supernatant of *L. acidophilus*, *B. bifidum* or *L. reuteri* to iDC during 20 h of culture did not induce cytokine production, indicating that the presence of intact bacteria is required for induction of IL-12 and IL-10 (Figure 2.2). Conversely, combining UV-killed *L. acidophilus* bacteria with sterile filtered supernatant of *B. bifidum* or *L. reuteri* diminished IL-12 production by DC markedly compared to the addition of *L. acidophilus* alone, suggesting that inhibitory components of these bacteria are released into the growth medium.

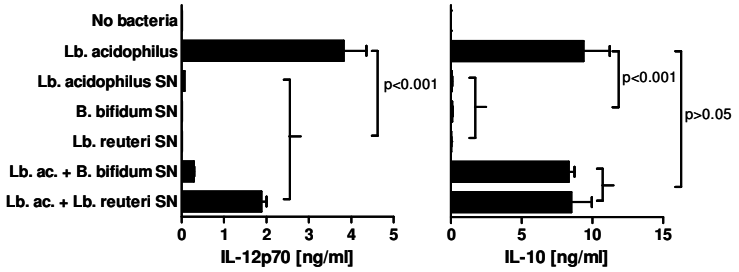

Figure 2.2: Induction of IL-12 production requires presence of bacteria, whereas inhibition of IL-12 production is induced by bacteria-derived soluble or shed factors. Sterile filtered supernatant (SN) from LAB grown in cell culture medium (*L. acidophilus*, *B. bifidum* or *L. reuteri*, 10% v/v) did not induce IL-12 or IL-10 production in iDC during 20 h of culture, but supernatant of *B. bifidum* and *L. reuteri* suppressed IL-12 production induced by UV-killed *L. acidophilus* (25 μg/10^6 DC/ml) without affecting IL-10 production. Data are means and SD of duplicate culture wells. Data are representative of three experiments with cells from different donors.

LAB with different cytokine-inducing properties deliver maturation stimuli to DC, and DC matured by LAB induce expression of activation markers on NK cells

Kinetics studies showed that peak *L. acidophilus*-induced IL-12 production occurred after only 6 hours of stimulation of DC with LAB (not shown). During this incubation period, all three strains of LAB increased expression of the maturation markers CD80, CD83, CD86 and HLA-DR on DC, *L. acidophilus* and *B. bifidum* more than *L. reuteri* (Figure 2.3). This indicates that low or absent IL-12 production by DC is not paralleled by a complete lack of maturation. Expression of HLA class I was slightly increased in DC after 6 hours of culture with LAB, and the expression increased further during prolonged incubation (Figure 2.3). The chemokine receptor necessary for migration to lymph nodes, CCR7, was absent on DC after 6 hours of culture with LAB but induced to a variable extent by the different LAB strains after 20 hours of culture (Figure 2.3).

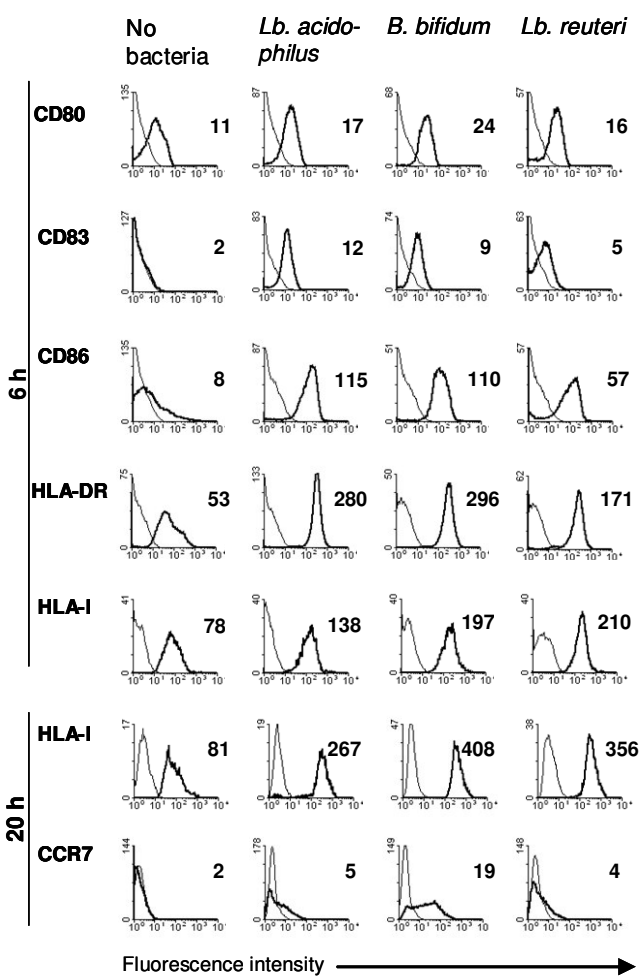

Figure 2.3: Short-term culture with LAB induces maturation of DC. Expression of surface maturation markers was measured after culture of iDC with no bacteria or with UV-killed LAB (*L. acidophilus*, *B. bifidum* or *L. reuteri*, 25 μg/10^6 DC/ml) for the indicated number of hours. Thin lines depict cells stained with an isotype control antibody; bold lines depict cells stained with an antibody specific for the surface marker indicated. Numbers in bold indicate the geometrical mean fluorescence intensity of cells stained with the specific antibody. Data are representative of four experiments with cells from different donors.

Since extensive upregulation of maturation markers occurred after only 6 hours of incubation with LAB, DC were matured for 6 hours (followed by washing to remove non-phagocytosed bacteria and secreted cytokines) for further studies of NK cell activation. DC matured by all three strains of LAB and combinations thereof induced expression of several surface markers indicative of NK cell activation (Figure 2.4A). Expression of CD25, the IL-2 receptor α-chain, indicates high responsiveness to IL-2. DC matured by all LAB caused upregulation of CD25 on NK cells, with *L. acidophilus*-matured DC being the most potent. CD56 is present on all resting NK cells, but to a lesser extent on $CD16^+$ NK cells, which is the more cytolytic subset of NK cells [141]. Upon activation of these $CD56^{dim}$ cells, CD56 is upregulated to the level present on resting $CD16^-CD56^{bright}$ NK cells. DC matured by all LAB efficiently upregulated CD56 on $CD56^{dim}$ NK cells. CD69 is a receptor for unknown ligands known as 'early activation marker' on NK cells and T cells [142]. CD69 was upregulated on NK cells cultured with all LAB-matured DC, but to a lesser extent by DC matured by *B. bifidum* or *L. reuteri*. DC matured by *B. bifidum* or *L. reuteri* combined with *L. acidophilus* also induced a slightly lower expression of CD69 in NK cells

than DC matured by *L. acidophilus* alone. HLA-DR is expressed on NK cells upon activation, and is possibly functional in antigen-presentation [143]. HLA-DR was upregulated on NK cells by LAB-matured DC, especially by DC matured by *B. bifidum* or *L. reuteri* both with and without the simultaneous presence of *L. acidophilus*. Finally, NKp44 is one of three identified orphan natural cytotoxicity receptors (NCRs) correlated with NK cell cytotoxicity, and the only NCR not constitutively expressed, but induced upon activation of NK cells [144]. All LAB-matured DC induced expression of NKp44 on a subset of NK cells.

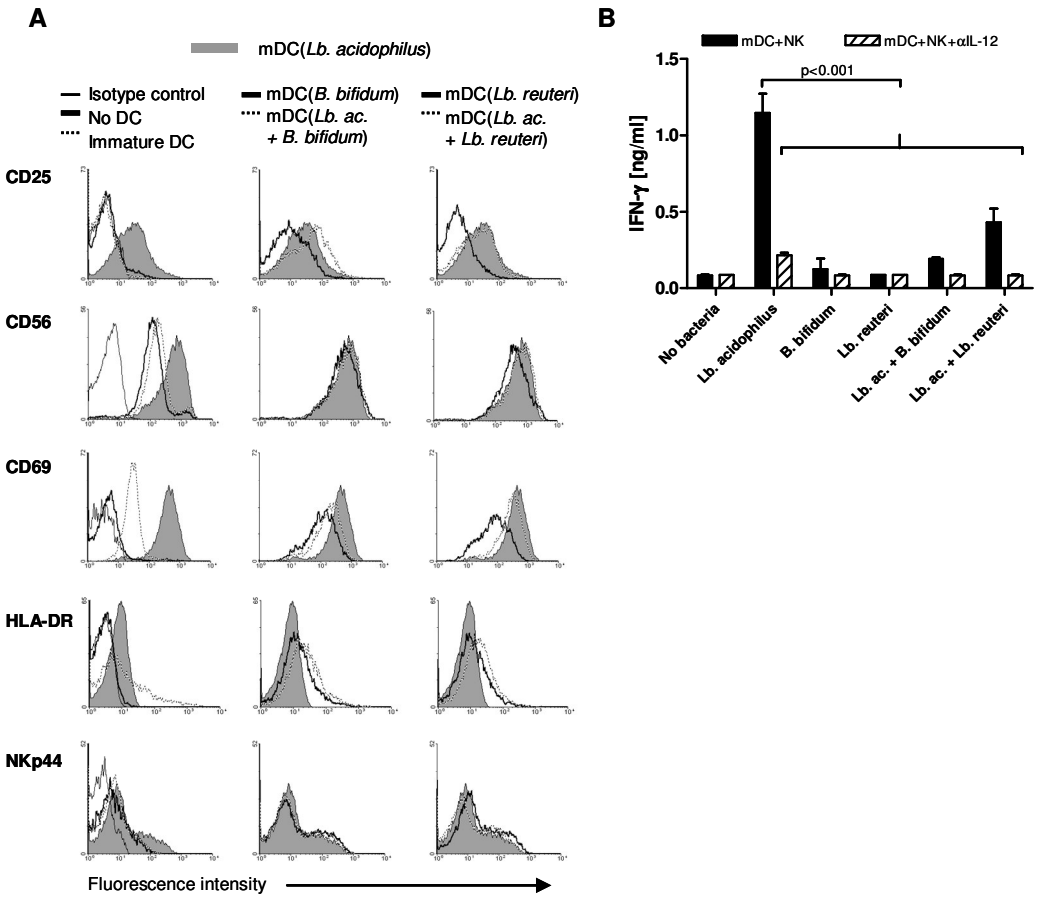

Figure 2.4: LAB-matured DC induce species-dependent activation and IFN-γ production in NK cells. (A) Expression of activation markers on NK cells was increased after culture with autologous DC matured for 6 hours with UV-killed LAB. In all panels, filled histograms depict NK cells cultured for 48 hours with DC matured by *L. acidophilus*. Thin lines depict cells stained with an isotype-matched control antibody, and bold or dotted lines depict NK cells cultured with or without different DC (iDC: immature DC, mDC: DC matured by the bacteria indicated) and stained with a antibody specific for the surface molecule indicated. Data are representative of two independent experiments. (B) NK cells (10^5/well) produced IFN-γ when incubated for 48 hours with autologous DC (mDC, $2 \cdot 10^4$/well) matured for 6 hours with UV-killed LAB (*L. acidophilus*, *B. bifidum*, *L. reuteri* or *L. acidophilus* combined with one of the other strains; 25 μg/10^6 DC/ml). IFN-γ production was reduced in the presence of an IL-12 neutralisation antibody. IFN-γ in culture supernatants was quantified by ELISA. Data are means and SD of duplicate cultures. Data are representative of four experiments with cells from different donors.

We next analysed whether six hours of contact with LAB allowed DC to modulate NK cell IFN-γ production. As expected, IFN-γ production was mainly induced in NK cells co-cultured with DC previously matured by *L. acidophilus* (Figure 2.4B), and neutralising IL-12 reduced IFN-γ production, indicating that IL-12 produced by mDC was largely responsible for induction of IFN-γ production in NK cells. Similarly to the simultaneous addition of iDC and LAB to NK cells (Figure 2.1B), addition of DC matured for 6 hours with either *B. bifidum*, *L. reuteri* or one of these combined with *L. acidophilus* induced less IFN-γ in NK cells than *L. acidophilus*-matured DC (Figure 2.4B).

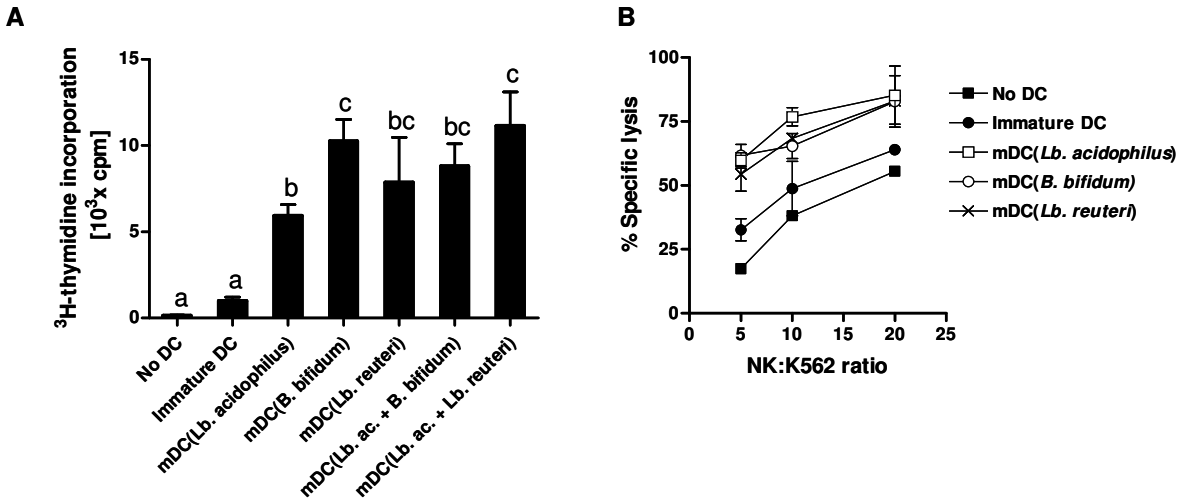

Figure 2.5: LAB-matured DC increase proliferation and cytotoxicity in NK cells. (A) NK cells (10^5/well) proliferated less when incubated for 4 days with autologous iDC ($2{\cdot}10^4$/well) than with mDC, DC matured for 6 hours with UV-killed LAB (*L. acidophilus*, *B. bifidum*, *L. reuteri*, or *L. acidophilus* combined with one of the other strains; 25 μg/10^6 DC/ml) followed by washing to remove bacteria (mDC, $2{\cdot}10^4$/well). Proliferation data are means and SD of cpm values of quadruplicate cultures and are representative of experiments with cells from three donors. Bars labelled with different letters indicate significantly different values ($P < 0.01$). Bars labelled with the same letters indicate that values are not significantly different ($P > 0.05$). (B) NK cells became more cytolytic after culture with DC. NK cells (10^6/well) were cultured for 48 hours in 48-well plates in the presence of autologous iDC or mDC (10^5/well) pre-matured by the indicated LAB strains for 6 hours (25 μg/10^6 DC/ml). Subsequently, 4 h cytotoxicity against the NK cell target K562 cell line was assessed by Cr51-release. Data are means and SD of triplicate measurements and are representative of three experiments with cells from different donors.

DC matured by all LAB strains expand NK cells and increase their cytolytic capacity

NK cell proliferation during 4 days of culture was increased by DC matured for 6 hours by the different strains of LAB and combinations thereof (Figure 2.5A). As previously described [134], mDC augmented proliferation significantly more than iDC. Mature DC alone or NK cells cultured separately with UV-killed bacteria did not proliferate (< 100 cpm, not shown). DC in general were seen to promote survival of NK

cells as the NK cell viability percentage was increased almost twofold in the presence of DC stimulated or not by bacteria (data not shown). The proliferation and viability measures suggest that bacterially matured DC increased the amount of viable NK cells in the cultures. In addition, DC matured by the different LAB strains increased NK cell cytotoxicity against the target cell line K562 after a 48 h co-culture of DC and NK cells, beyond the increase seen with iDC (Figure 2.5B).

Discussion

LAB are known to be involved in the maintenance of gut immune homeostasis, and now also emerge as potential NK cell modulators. All LAB strains tested in the present study mediated, via DC maturation, expansion of NK cells and increased their cytotoxic efficacy as well as the level of cytotoxicity-related activation markers. This indicates that LAB, similarly to pathogenic microorganisms and inflammatory stimuli, license DC to signal to NK cells. Our data suggest that stimulation of NK cell proliferation and cytotoxicity is a general ability of LAB. In support of this finding, the increased NK cell activity shown in mice and humans upon consumption of lactobacilli and bifidobacteria was obtained using a number of different species and strains [114, 127-129, 139]. An enlarged and more cytolytic pool of NK cells would be beneficial prophylactically in healthy individuals, but also therapeutically in most pathologies. Moreover, as NK cell cytotoxicity is tightly controlled by the presence of NK cell receptor ligands on target cells [19], an expansion of NK cells caused by gut LAB is not likely to cause excess tissue damage.

In the present study, IL-12 production in DC was strongly induced by *L. acidophilus*, whereas all LAB tested induced substantial amounts of IL-10. Other LAB strains have been found to elicit highly variable levels of these cytokines [85, 100] Only DC matured by *L. acidophilus* induced high amounts of IFN-γ in NK cells, suggesting that not all LAB have this capability. It is generally accepted that IL-12 induces IFN-γ production in human NK cells [137], as it is also the case in our study. Recent findings indicate that IFN-γ production by NK cells is required to induce Th1 responses in lymph nodes [25], emphasising the importance of bacterial regulation of IL-12 production in DC. It is not known how *L. acidophilus* induces IL-12 production in DC. However, as *L. acidophilus* supernatant was unable to induce IL-12 production, this stimulation is hypothesised to involve a cell wall component, possibly acting via TLRs on DC. This would be in accordance with Michelsen et al. [145] showing that maturation and IL-12 production can be induced in murine DC via TLR2 recognising PG and LTA. Amounts of TLR2 ligands expressed by the different LAB may be the reason for the observed differences in induction of maturation surface markers in monocyte-derived DC cultured with LAB for 6 hours. We have observed that bifidobacteria are generally poor inducers of DC maturation [84], but within this short time frame *B.*

bifidum matured monocyte-derived DC to the same extent as *L. acidophilus*. In agreement with our previous results [83, 84], *B. bifidum* and *L. reuteri* were found to inhibit *L. acidophilus*-induced IL-12 production in DC, and accordingly abrogated IFN-γ production by NK cells. A similar mechanism may cause a probiotic mixture of 8 strains of LAB, VSL#3, to induce only IL-10 production in murine DC [146]. This dominant IL-12-inhibitory property of a mixture of LAB may be of importance in the intestine where thousands of strains co-exist. The inhibitory components of these bacteria are seemingly secreted or "shed" into the medium during growth, perhaps indicating that such compounds reach gut DC compartments that intact bacteria do not normally access. In our study, we found that IL-10 played a role in limiting the amount of IL-12 produced in response to LAB, as neutralising IL-10 increased the IL-12 production induced by all single and combined LAB strains. It is known that IL-10 inhibits IL-12 production in DC in an autocrine fashion by regulating IL-10 receptor expression and activating STAT-3 [147]. Nevertheless, IL-10 was not responsible for the inhibition of *L. acidophilus*-induced IL-12 production by *B. bifidum* and *L. reuteri*, as this inhibition was also evident in the presence of IL-10-blocking antibody, and when *B. bifidum* and *L. reuteri* were replaced by their non-IL-10-inducing supernatant. Therefore it is unlikely that the secreted components of *B. bifidum* and *L. reuteri* exerting the IL-12 inhibitory effect are merely strong inducers of IL-10. Probably, they interact directly with inhibitory receptors on DC, such as dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) recognising carbohydrate structures expressed by e.g. *Helicobacter pylori* and mycobacteria, enabling their immune escape [79].

DC matured by the different strains of LAB all induced activation markers in NK cells. CD56 and NKp44 were induced to a comparable extent by all strains, whereas CD25 was induced to a greater extent by *L. acidophilus* and HLA-DR by *B. bifidum* and *L. reuteri*. Similarly to the IFN-γ production, the level of CD69 expression on NK cells seemed dominated by *B. bifidum* and *L. reuteri* when these bacteria were present together with *L. acidophilus* during maturation of DC, as CD69 expression was reduced in NK cells stimulated with DC matured by *L. acidophilus* together with *B. bifidum* or *L. reuteri*, compared to NK cells stimulated by DC matured by *L. acidophilus* alone. Expression of CD69 has been shown to correlate with NK cell cytotoxicity [148], but its expression on NK and T cells has also been implicated in immune down-regulation [149], possibly underlining the regulatory properties of *B. bifidum* and *L. reuteri*. Regarding HLA-DR expression on NK cells, combining two strains of bacteria for DC maturation yielded a small additive effect, but as the role of NK cells as antigen-presenting cells remains ill-defined it is difficult to interpret the functional significance of such differences. Presumably, the cytokines and NK cell ligands expressed by LAB-matured DC, which are responsible for induction of

activation markers, increased cytotoxicity, and proliferation in NK cells, do not differ importantly from NK activating molecules secreted by DC matured with LPS or pro-inflammatory cytokines. These include type I interferons, IL-2, IL-15, IL-18, CD40 and CD80/CD86 (reviewed in [150, 151]).

In this study, we observed no direct stimulation of highly purified NK cells by any of the LAB analysed in the absence of DC. NK cells have previously been shown to be the lymphocyte population most sensitive to activation by LAB, but only in the presence of monocytes [80, 152]. These observations, however, do not rule out that NK cells in the gut may be able to directly detect LAB, possibly by interaction between bacterial CpG DNA and TLR9, which is present in NK cells [27, 153]. We addressed this by testing the direct effect of LAB-stimulation on both resting and polyclonally activated NK cells, but observed no IFN-γ production (not shown), so the LAB used in this study seemingly do not interact with TLR9 on NK cells, but rather with TLR2 on accessory cells as discussed above. TLR2 has been shown to be absent or expressed in very low amounts in NK cells [154, 155], and its ligation only activates NK cells in the presence of exogenous IL-12 [155].

We chose the shortest time-period of LAB exposure yielding mature DC, 6 hours, for the co-culture experiments. However, CCR7 was not upregulated until after 20 hours of maturation by LAB. This may indicate that migration of DC from the lamina propria or Peyer's patches to mesenteric lymph nodes does not occur before prolonged exposure to LAB or simultaneous exposure to other maturation stimuli have taken place. HLA-I molecules may protect against lysis by NK cells during migration, as HLA-I deficient DC are efficiently killed by autologous NK cells, at least in vitro [133]. Also in this study, we observed that more immature DC than LAB-matured DC were lysed by activated NK cells (not shown). Lymph nodes have been identified as one of the sites where NK cells and DC encounter [25, 35, 137], and it is likely that gut-derived DC reach mesenteric lymph nodes upon LAB stimulation and acquisition of CCR7, and engage in cross-talk with resident NK cells in the paracortex, where concomitant $CD4^+$ T cell activation takes place [35, 137]. Therefore, NK cells interacting with migrating DC may regulate co-localised T cell responses.

In conclusion, LAB potently initiate NK-DC interactions via DC maturation. NK cells expand and increase their cytolytic potential. The balance between NK cell responses and regulatory responses may prove delicately regulated by intestinal LAB, as NK cell effector functions are subjected to suppression mediated by Tregs [20], and these Tregs may be induced by LAB [99], which at the same time sustain NK cell cytolytic activity. However, in contrast to other DC maturation stimuli, different LAB have varying effects on NK cell IFN-γ production. Since Th1-promoting LAB can easily be identified in this model system, these LAB may represent a useful tool for modulating the cytokine balance during

autoimmune diseases driven by ThIL-17 cells in the absence of IFN-γ [156], and to promote potent type 1 immune responses, desirable in infection and cancer. In addition, the presence of LAB early in life skewing the immune system towards a Th1 response, possibly through the intermediate of NK cells, may aid in the prevention of Th2-mediated allergy. Finally, the evidence that weak IFN-γ-inducing LAB strains are able to suppress the action of IFN-γ-inducing strains while preserving NK cell stimulatory activity, could represent a pivotal mechanism in maintaining immunological homeostasis in the intestine in the absence of infection.

Acknowledgements

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3. Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation

In collaboration with Louise H. Zeuthen, Guido Ferlazzo and Hanne Frøkiær.

Abstract

The intestinal microbiota is essential for development and homeostasis of the local and systemic immune system, and particularly strains of lactic acid bacteria and Escherichia coli have been shown to have balancing effects on inflammatory conditions such as allergy and inflammatory bowel disease. However, distinct strains possess different potential to polarise and regulate the immune response, and in vitro results used to choose strains for therapeutic purposes may depend strongly on the cell type used as a model. To select the most appropriate model for screening of beneficial intestinal bacteria in human cells, we compared the response to strains of intestinal bacteria of three types of antigen-presenting cells (APC); blood myeloid dendritic cells, monocyte-derived dendritic cells and monocytes, and characterised the effector response of natural killer cells and naïve T cells. The response to bacteria was markedly different between APC as concerns maturation and induction of pro-inflammatory cytokines, with blood dendritic cells and monocytes responding with production of interleukin-6 and tumour necrosis factor-α to bacteria, which elicited IL-10 production in monocyte-derived dendritic cells. In contrast, an interferon-γ inducing capability of all APC cultured with specific bacterial strains and an interferon-γ-inhibitory capability of other strains were generally observable, and in both natural killer cells and T cells, with the most potent responses induced by monocyte-derived dendritic cells, which thus constitute the most sensitive screening model.

Introduction

Intestinal bacteria promote the early development of the host immune system, and contribute to appropriate balancing of immune responses later in life. These interactions, which have not been fully characterised, involve both epithelial cells and cells of the immune system [45]. Germ-free mice have a reduced number of $CD4^+$ T cells and dendritic cells (DC) compared to colonised animals [69, 72]. Moreover, the establishment of the intestinal microbiota is presumed responsible for polarising the immune system away from the Th2 profile of the neonate [157]. Both infectious bacteria and enteric commensals are seemingly involved in this early polarisation, and data suggest that pattern recognition

receptors (PRRs) of epithelial cells and immune cells are able to cooperate in intricate ways to distinguish molecules expressed by the majority of bacteria from molecules associated with pathogenicity [158, 159]. In addition to epithelial cells, DC is presumed the major cell type responsible for sensing the intestinal microbiota as they express a plethora of PRRs and are capable of projecting dendrites through the epithelial layer to sample the intestinal content [60]. Moreover, DC are the only antigen-presenting cells (APC) capable of inducing regulatory T cells, necessary for establishment of the tolerogenic environment of the intestine [160].

The effect of gut-associated lactic acid bacteria (LAB, used here to designate lactobacilli and bifidobacteria) on the immune system has been studied extensively in vitro, in animal disease models and randomised human trials. Emphasis in both in vitro and in vivo experiments has been on the ability of LAB to increase innate immune function, including phagocytosis and natural killer (NK) cell cytotoxicity [127, 138, 161], and to polarise T cells through cytokine induction [85, 99, 110]. Immunomodulatory effects of LAB are highly strain-dependent, with some strains inducing the type 1 polarising cytokine interleukin (IL)-12 [85] and other strains inducing high amounts of IL-10, which promote regulatory T cell responses [99]. In humans, the outcome of supplementation with specific strains of LAB has been prevention of acute diarrhoea [124], alleviation of atopic eczema [119] and certain types of inflammatory bowel disease [102, 105], indicating stimulation of the immune system and/or normalisation of an aberrant immune response. However, in some recent clinical trials probiotic intervention has proven unsuccessful [109, 118], and careful strain-selection may be a way to better target specific immune-mediated diseases.

It is of major interest to have simple model systems wherein the immunomodulatory effects of different bacterial strains can be evaluated to appreciate strain-dependent characteristics of commensal bacteria and to select the appropriate strains for prevention and treatment of diseases. In studies using in vitro generated monocyte-derived DC (MoDC) we have observed marked differences between on one hand strong IL-12-inducing *Lactobacillus* strains and on the other hand other *Lactobacillus* strains and strains of *Bifidobacterium* that are poor IL-12-inducers and in addition capable of inhibiting IL-12 production induced by one of the first-mentioned *Lactobacillus* strains [84]. *Escherichia coli* are, similarly to *E. coli* LPS, poor IL-12-inducers in our DC models, but, as opposed to poor IL-12-inducing LAB, strong inducers of maturation in DC [84].

Other groups have studied the cytokine-inducing properties of gut-derived bacteria in human blood monocytes (Mo) [80, 162], whereas the effect of gut-derived bacteria on blood DC has not been reported. Comparisons of Mo, blood myeloid DC (BDC) and MoDC have revealed differences in the

types and levels of PRRs expressed [163], and in vitro studies in Mo and DC may therefore lead to different conclusions on the same bacterial strains. These cell populations also differentially stimulate T cells [164, 165], and may represent different accessory functions relevant in vivo. The aim of this study was to select an appropriate model for screening of potential probiotic bacterial in human cells. MoDC have already proven useful, but as Mo are more readily available from a large number of test persons they would provide a simpler test system. On the other hand, BDC may reflect in vivo DC populations better that MoDC, but the former are difficult to obtain in large numbers. In an attempt to deduce general properties of the bacteria across APC types, we compared the response of these three types of human APC to different gut-derived strains of bacteria, including two Lactobacillus strains, one Bifidobacterium strain and the commensal E. coli strain Nissle 1917. We have previously found that LAB-matured MoDC activate NK cells to kill target cells, proliferate and secrete cytokines, but only the cytokine response was strain-dependent (Chapter 2). Here we studied whether gut-derived commensals prime BDC and Mo similarly to induce NK cell cytokine production. The analysis of the four gut bacteria combined with different APC also included their interaction with allogeneic naïve T cells, to assess whether inhibition of IL-12 production in APC by specific LAB would translate into reduced IFN-γ production and lower numbers of Th1 cells.

We observed major differences in the induction of surface maturation markers and TNF-α and IL-6 production in different APC stimulated with individual bacteria, but a pronounced similarity in inducing the Th1 polarising cytokine IL-12. Interferon (IFN)-γ production was induced in NK cells and naïve T cells by all APC when IL-12 was produced, and bacterial inhibition of IL-12 production in APC translated into reduced IFN-γ production in both NK cells and T cells for all APC.

Materials and methods

Bacterial strains

The bacterial strains used were: *L. acidophilus* X37, *B. longum* Q46 (both isolated from adult intestinal biopsies and typed at the Department of Food Science, University of Copenhagen, Denmark), *L. reuteri* DSM 12246, and *E. coli* Nissle 1917. Lactobacilli and bifidobacteria were grown in de Man, Rogosa and Sharpe broth overnight and *E. coli* were grown in Luria-Bertani broth overnight (media were from Merck, Darmstadt, Germany). Bacterial cells were washed twice in sterile PBS and UV-irradiated in a thin film of liquid in Petri-dishes for 10 min. Bacterial suspensions were diluted in complete cell culture medium and added to cell culture on a dry-matter basis. Endotoxin contamination of suspensions of Gram-positive

bacteria was tested with the Pyrochrome kit (Ass. of Cape Cod, East Falmouth, MA) and was < 0.015 endotoxin units/ml.

Isolation and culture of cells

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats (Blodbanken, Copenhagen University Hospital) by Ficoll-Paque centrifugation (Amersham, Uppsala, Sweden). $CD14^+$ Mo were isolated using MACS CD14-microbeads (Miltenyi, Bergisch Gladbach, Germany) and MoDC were generated with addition of GM-CSF and IL-4 as previously described [84]. Mo and MoDC contained less than 5% contaminating lymphocytes. BDC were isolated from the remaining PBMC using the MACS $CD1c^+$ DC isolation kit (Miltenyi). BDC were 75-80% HLA-DR^{high} cells and devoid of $CD14^+$ cells. NK cells were isolated from PBMC using the MACS NK cell isolation kit (Miltenyi) and were consistently > 90% $CD3^-CD56^+$ cells. Naïve $CD4^+$ T cells were isolated using the MACS Naïve T cell isolation kit (Miltenyi) and were > 95% $CD3^+CD4^+CD45RA^+$. Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in complete medium (RPMI 1640 supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, all from Cambrex Bio Science, East Rutherford, NJ). APC ($2 \cdot 10^4$ cells/well or $4 \cdot 10^4$ cells/well) were cultured with bacterial suspensions for 18 hours in round-bottom 96-well plates. To some 18 h APC cultures ($2 \cdot 10^4$ cells/well) autologous NK cells were added (10^5 cells/well). APC ($2 \cdot 10^4$ cells/well) were co-cultured with allogeneic naïve $CD4^+$ T cells at a ratio of 1:5 for 6 days. Supernatants were harvested and T cells re-stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin (both Sigma, St. Louis, MO) for 6 hours in the presence of brefeldin A (GolgiPlug, BD Biosciences, Franklin Lakes, NJ) for detection of intracellular cytokines. To some APC-effector cell cultures 10 μg/ml of an IL-12 neutralisation antibody (R&D Systems, Minneapolis, MN) was added. An overview of isolation and culture of cells is given in Figure 3.1.

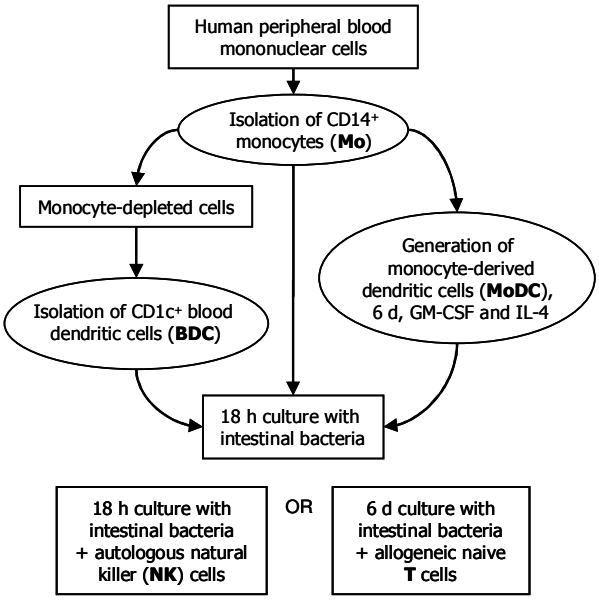

Figure 3.1: Isolation/generation of three types of APC and co-culture with NK cells and naïve T cells. Three types of APC were isolated or generated from the same donor to compare the response of different APC to intestinal bacterial isolates. Autologous NK cells were added to some APC-cultures. NK cells cultured with MoDC were isolated from cryopreserved autologous PBMC, as the generation of MoDC requires six days of culture. Naïve T cells were isolated from cryopreserved PMBC and added to allogeneic APC-cultures.

Cytokine determinations by ELISA

Cytokine content in cell culture supernatants was determined using commercial antibody pairs and recombinant standards for tumour necrosis factor (TNF)-α, IL-12p70, IL-6, IL-4 (DuoSet, R&D Systems), IL-10 (BD Biosciences, clones: unlabelled JES3-19F1 and biotinylated JES3-12G8) and IFN-γ (CytoSet, Biosource, Camarillo, CA).

Flow cytometric analysis

Flow cytometric analysis was conducted on a FACSArray flow cytometer (BD Biosciences). For phenotyping of the isolated cells the following antibodies were used: allophycocyanin-conjugated anti-CD4, allophycocyanin-Cy7 conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD45RA (eBioscience, San Diego, CA), PE-conjugated anti-CD56 (Miltenyi), PE-conjugated anti-CD14 (Caltag/Invitrogen, Carlsbad, CA), and PE-conjugated anti-HLA-DR (Southern Biotech, Birmingham, AL). Analysis of APC surface marker expression was performed using PE-conjugated antibodies against CD80 and HLA-DR and allophycocyanin-conjugated anti-CD40 (all Southern Biotech) and anti-CD83 (BD Biosciences). Anti-CD25-PE and anti-CD69-PE for analysis of activation markers were purchased from eBioscience and Caltag, respectively. Intracellular staining of T cells was performed with antibodies from BD Biosciences (anti-IL-10-PE) and eBioscience (anti-IFN-γ-PECy7 and anti-IL-4-PE). Prior to intracellular staining, T cells were labelled with anti-CD4-allophycocyanin, fixed in 4% methanol-free formaldehyde (Polysciences Inc., Warrington, PA) and permeabilised in PBS containing 0.1% saponin and 0.5% bovine serum albumin (both from Sigma).

Statistical analysis

Statistical analysis (one-way analysis of variance) was performed using the GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA). Differences were considered significant if $p < 0.05$. Data shown are representative of minimum three independent experiments with different donors.

Results

Different gut-derived bacteria induce variable levels of HLA-DR and co-stimulatory molecules in different types of APC

Expression of certain surface molecules (HLA-DR, CD80, CD86, CD83 and CD40) on APC is indicative of the level of maturation of the cells. Most of these maturation markers have known ligands on T cells, and are therefore predictive of the quality of the APC-T cell interaction during initiation of an immune response. Unstimulated, MoDC and BDC showed a high expression of HLA-DR and co-stimulatory molecules compared to unstimulated Mo. HLA-DR and CD40 were expressed to a greater extent on MoDC than on BDC, whereas for CD80 and CD83 the opposite was the case (Figure 3.2).

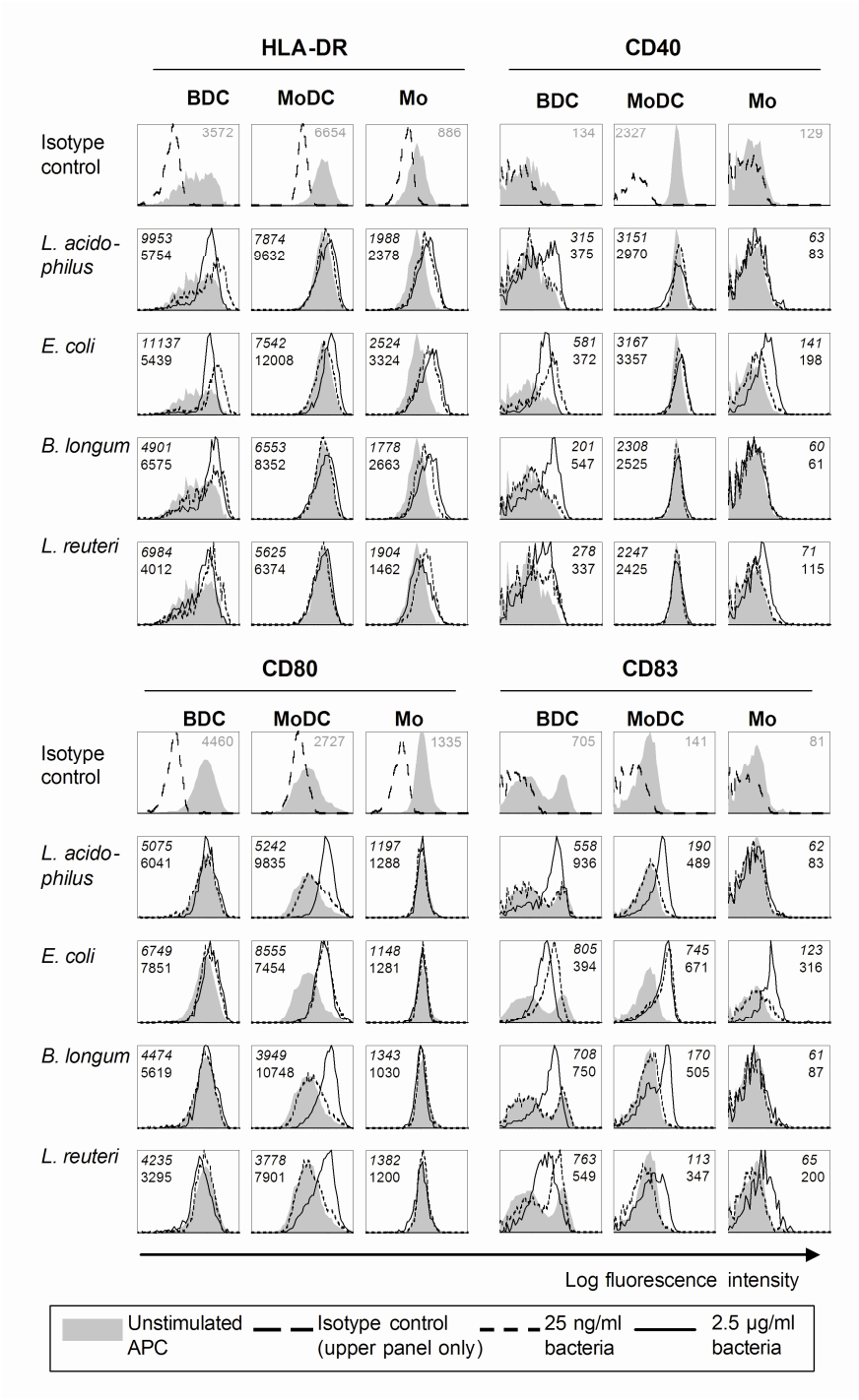

Figure 3.2: Expression of HLA-DR and co-stimulatory molecules in response to gut-derived bacteria differs between types of APC. Different APC ($2 \cdot 10^4$ cells/0.2 ml/well) were incubated for 18 hours in the presence of UV-killed gut-derived bacteria and stained for surface markers indicative of maturation. Numbers in grey indicate the geometrical mean fluorescence intensity of unstimulated cells. Italic and non-italic letters indicate the geometrical mean fluorescence intensity of cells stimulated with 25 ng/ml and 2.5 µg/ml UV-killed bacteria, respectively.

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Analysis of the expression of surface markers on APC after in vitro stimulation with gut-derived bacteria revealed that all bacteria altered the expression of all surface markers on both kinds of DC, except *B. longum* and *L. reuteri* that did not affect the expression of HLA-DR and CD40 on MoDC, and CD80 on BDC. *L. acidophilus*, *E. coli* and *B. longum* were generally more potent in inducing DC-maturation markers than *L. reuteri*. The low dose (25 ng/ml) of *E. coli* was as efficient as 2.5 µg/ml in

inducing co-stimulatory molecules on DC, whereas only the high dose of the three strains of LAB upregulated these markers on DC. Although maturation markers were upregulated on BDC by bacterial stimuli, MoDC consistently expressed higher levels of HLA-DR and co-stimulatory molecules. Only CD83 was expressed to a comparable extent by BDC and MoDC after stimulation. All bacteria also modulated the expression of HLA-DR on Mo, but only the high dose of *E. coli* or *L. reuteri* increased the expression of CD40 and CD83, and CD80 expression on Mo was not affected by bacterial stimulation. Expression of all maturation markers on Mo after bacterial stimulation remained lower than the expression on DC.

Gut-derived bacteria induce IL-12- and IL-10 production primarily in MoDC, and strain-dependent levels of pro-inflammatory cytokines IL-6 and TNF-α in all types of APC

When APC were stimulated with titrated doses of UV-killed gut-derived bacteria, MoDC were the most potent cytokine-producers, with the highest amount of TNF-α and IL-12p70 induced by 2.5 μg/ml of *L. acidophilus* and the highest amounts of IL-6 and IL-10 induced by *E. coli* (Figure 3.3A). *E. coli* was the only strain inducing cytokine production in APC at the lowest concentration tested (25 ng/ml). Analysis of the pro-inflammatory cytokines TNF-α and IL-6 showed a difference in the response of BDC and MoDC to the different strains. Of note, *L. reuteri* was the poorest inducer of pro-inflammatory cytokines in MoDC, but in BDC *L. reuteri* induced the highest amount of inflammatory cytokines among the LAB, and *L. reuteri* was also an effective inducer of pro-inflammatory cytokines in Mo.

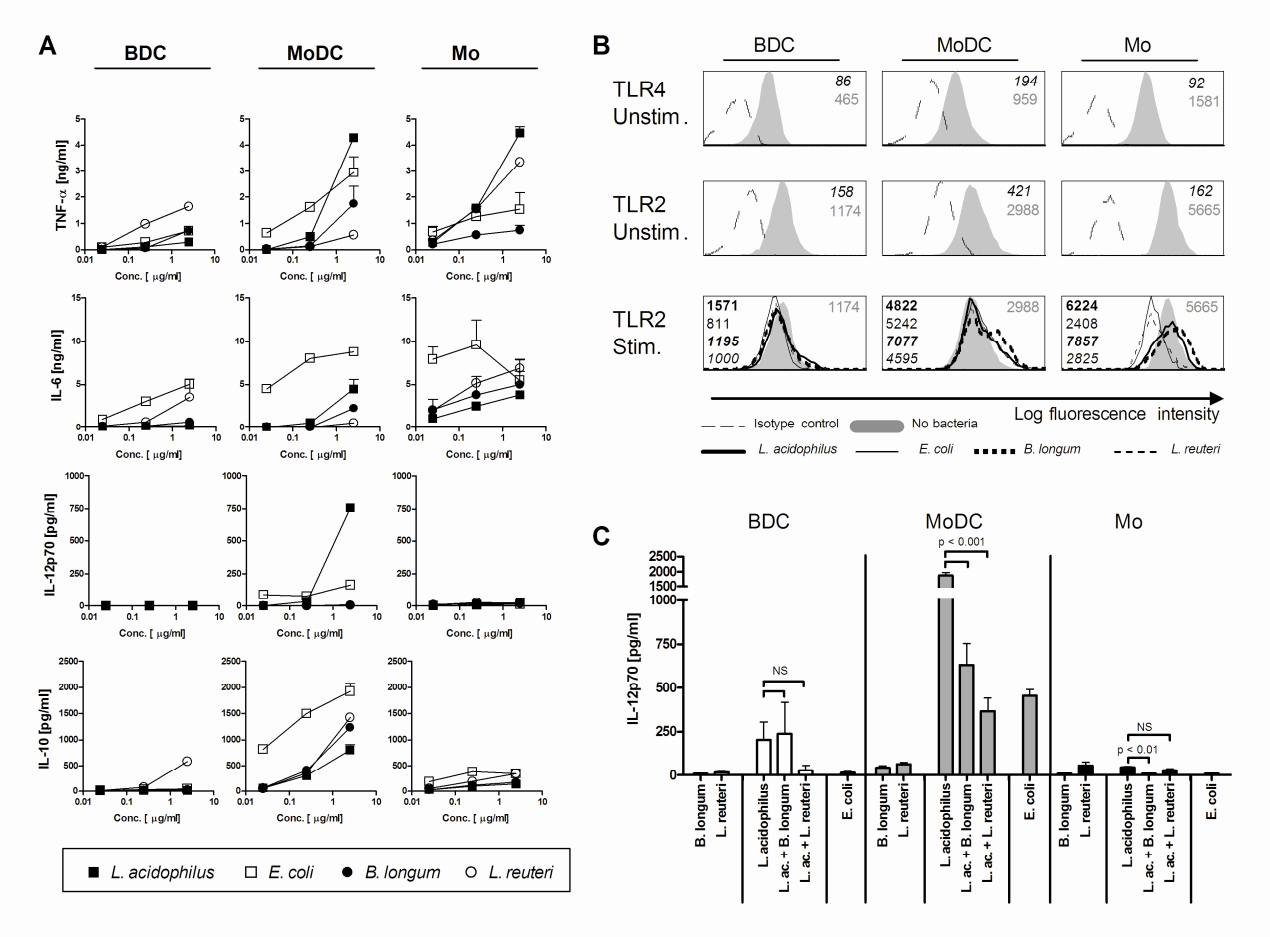

Figure 3.3: Gut bacteria induce varying cytokine patterns in three types of APC differentially expressing Toll-like receptors. A. Different APC ($2 \cdot 10^4$ cells/0.2 ml/well) were incubated for 18 hours in the presence of UV-killed gut-derived bacteria and supernatant cytokine levels determined by ELISA. Data points depict means and SD of duplicate culture wells. B. TLR4 and TLR2 expression on APC was measured by flow cytometry. Numbers in italic indicate the geometrical mean fluorescence intensity of the isotype-stained cells and numbers in grey the geometrical mean fluorescence intensity of specifically stained cells. In the last panel, TLR expression on bacterially stimulated cells is compared to unstimulated cells (filled histogram), with numbers indicating the geometrical mean fluorescence intensity of cells stimulated with individual bacterial strains (Bold: *L. acidophilus*, non-bold: *E. coli*, bold italic: *B. longum*, non-bold italic: *L. reuteri*). C. Cytokine production by APC ($4 \cdot 10^4$ cells/0.2 ml/well) cultured for 18 hours in the presence of 5 μg/ml of single or paired strains of UV-killed gut-derived bacteria. Bars depict means and SD of duplicate culture wells. 'NS' indicate not significant.

The expression of TLR2 and TLR4 on the surface of the APC was assessed to relate the cytokine response to the potential binding of Gram-positive and Gram-negative bacteria to TLR2 and TLR4, respectively. Both receptors were expressed by all APC (Figure 3.3B), to the greatest extent on Mo, and slightly more on MoDC than on BDC. Interestingly, stimulation of BDC and Mo with *E. coli* or *L. reuteri* reduced TLR2 expression, whereas *L. acidophilus* and *B. longum* slightly increased TLR2

expression. All bacteria increased TLR2 expression on MoDC. None of the bacteria altered TLR4 expression on APC (not shown).

Bacterially induced IL-12 production is inhibited by bacteria, which are poor IL-12-inducers

L. acidophilus and *E. coli* induced detectable amounts of IL-12 in MoDC, whereas IL-12 was produced in very low amounts in BDC and Mo under the conditions used in Figure 3.3A. Increasing cell and bacterial numbers (Figure 3.3C) led to a detectable IL-12 production in BDC and Mo stimulated with *L. acidophilus,* and in Mo stimulated with *L. reuteri* but not with *B. longum* or *E. coli*. Only *L. reuteri* induced IL-10 in BDC, whereas all bacteria induced some IL-10 production in MoDC and Mo. Work from our group has shown that IL-12p70 production induced in MoDC by *L. acidophilus* can be significantly reduced if strains of *L. reuteri* or *B. longum* are added simultaneously to the cells [84]. In this study, the effect of *L. reuteri* or *B. longum* on *L. acidophilus*-induced IL-12 production was tested also in BDC and in Mo (Figure 3.3C). As opposed to MoDC, neither *B. longum* nor *L. reuteri* inhibited *L. acidophilus*-induced IL-12 production in BDC significantly. *B. longum* and *L. reuteri*, as expected, inhibited IL-12 induction by *L. acidophilus* in MoDC, whereas in Mo only *B. longum* reduced IL-12 production significantly. *B. longum* and *L. reuteri* did not reduce IL-12 production induced by *E. coli* regardless of APC type (not shown).

Gut bacteria-activated APC induce IL-12 dependent IFN-γ production and CD69 expression in autologous NK cells

When different APC were cultured with UV-killed gut-derived bacteria and freshly isolated autologous NK cells, the IL-12 production induced in APC by especially *L. acidophilus* was accompanied by IFN-γ production in NK cells (Figure 3.4A). IFN-γ production was not detected in APC or NK cells cultured separately with the bacterial stimuli (not shown). *B. longum* or *L. reuteri* added to the APC-NK co-cultures together with *L. acidophilus* markedly reduced the amount of IFN-γ generated by NK cells compared to cultures stimulated with *L. acidophilus* alone, and *L. acidophilus*-induced IFN-γ production was also effectively reduced upon addition of an anti-IL-12 neutralisation antibody for all types of APC (Figure 3.4B). In line with its modest stimulation of IL-12 production in APC, IFN-γ was not potently induced by *E. coli*. CD69 is a surface marker of activated lymphocytes, and its expression correlates with cytolytic activity in NK cells [166]. Expression of CD69 was slightly increased when freshly isolated NK cells were cultured with MoDC, but only markedly increased when NK cells were cultured with both APC and intestinal bacteria (Figure 3.4C). BDC and Mo together with bacteria increased CD69 levels considerably

less than bacterially stimulated MoDC. *L. acidophilus* and *E. coli* increased the CD69 expression in NK cells the most with all types of APC.

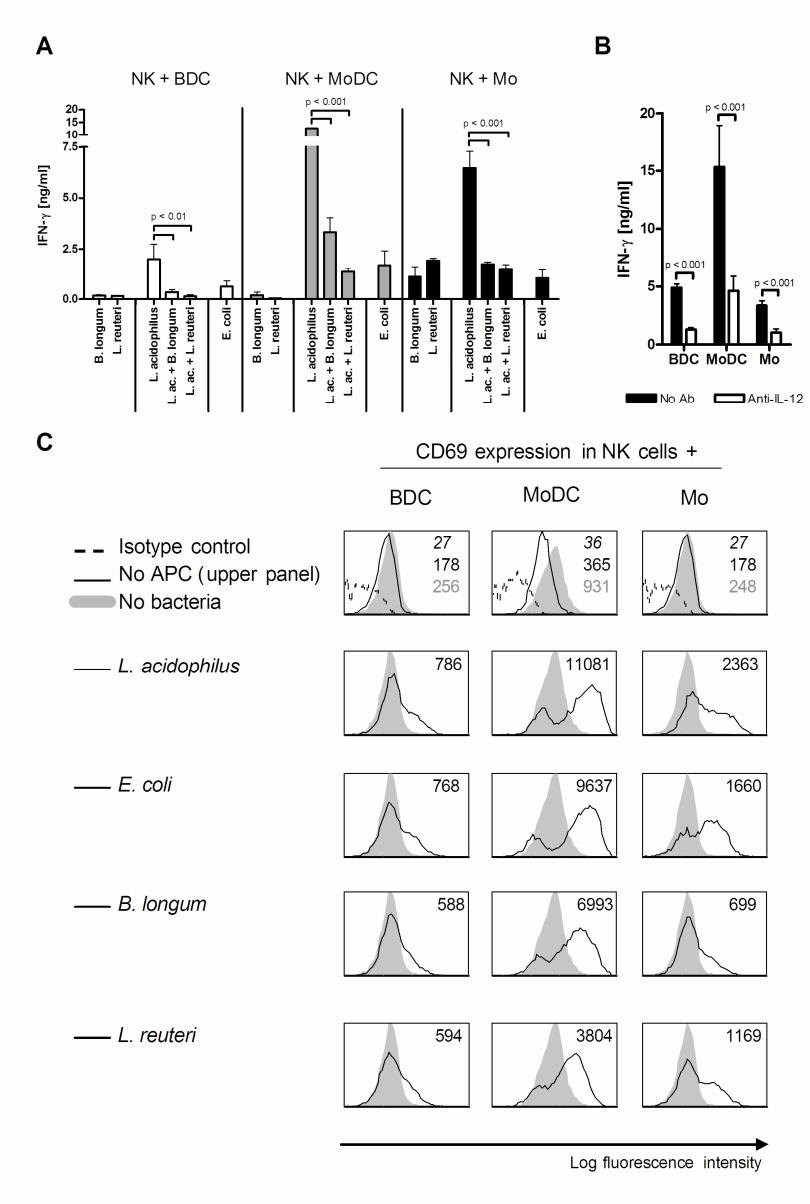

Figure 3.4: Culture with APC and gut bacteria induces IL-12-dependent IFN-γ production and CD69 expression in NK cells. A. Cytokine production by NK cells co-cultured with three types of APC and gut-derived bacteria (2.5 µg/ml). Bars depict means and SD of duplicate culture wells. B. Cytokine production by NK cells co-cultured APC in the presence UV-killed gut-derived *L. acidophilus* and anti-IL-12 antibody. Bars depict means and SD of duplicate culture wells. C. CD69 expression on NK cells after co-culture with APC and UV-killed gut-derived bacteria. Numbers in grey indicate the geometrical mean fluorescence intensity of NK cells cultured with APC but without bacteria. Italic letters indicate the geometrical mean fluorescence intensity of the isotype-stained cells. Non-italic letters indicate the geometrical mean fluorescence intensity of NK cells cultured without APC (upper panel only) or with APC and bacteria.

APC cultured with gut-derived bacteria induce strain-dependent levels of IFN-γ production in allogeneic naïve T cells

The T cell polarising potential of the four LAB and *E. coli* strains mediated by different types of APC has not been compared before. IFN-γ and IL-10 content was determined in the supernatant of naïve T cells cultured with allogeneic APC and single and combined bacterial strains, and intracellularly upon non-specific restimulation with PMA and ionomycin. Both types of DC generated stronger allogeneic IFN-γ

responses than Mo, but the strain-dependent pattern of IFN-γ induction was similar across APC types (Figure 3.5A). *L. acidophilus* and *E. coli* induced the highest amount of IFN-γ production in T cells, both during the allogeneic stimulation and after re-stimulation. *B. longum* and *L. reuteri* induced less IFN-γ in the supernatant, and reduced IFN-γ production when present together with *L. acidophilus,* but without reducing the number of IFN-γ-producing cells. Although the percentage of IFN-γ T-cells was not much higher, cells stained more brightly for IFN-γ when stimulated with APC and L. acidophilus and E. coli than with no bacteria, B. longum or L. reuteri (illustrated for MoDC in Figure 3.5B). IFN-γ production was partially reduced when cells were stimulated with DC, but not Mo, in the presence of IL-12 neutralisation antibody (Figure 3.5C). All bacteria induced IL-10 in the APC-T cell supernatant, but IL-10 was primarily produced by DC (not shown). IL-10 was detected intracellularly in less than 1% of the T cells stimulated with LAB and APC, and the IL-10 response to different bacteria was highly donor dependent (not shown). IL-4 was never detected intracellularly, only in very low amounts in the culture supernatants, and no differences were observed between strains of bacteria (not shown). Naïve allogeneic T cells were potently activated by DC both in the presence or absence of gut bacteria, but by Mo only without bacteria or with *L. acidophilus,* as can be seen from their expression of CD25 (Figure 3.5C). CD25 expression was not affected by neutralisation of IL-12 (not shown). Naïve T cells stimulated by different bacteria in the absence of APC were not activated and did not produce IFN-γ (not shown).

Discussion

The investigation of the responses of different types of APC to selected strains of gut-derived bacteria showed marked differences in upregulation of maturation markers and in the induction of pro-inflammatory cytokines. In contrast, the IFN-γ-inducing capability of APC cultured with *L. acidophilus* and the IFN-γ-inhibitory capability of APC cultured by *L. reuteri* and *B. longum* were seen with all types of APC, and in both NK cells and T cells.

The differential upregulation of maturation-associated surface markers on APC by different gut-derived bacteria reflects the different immunomodulatory potential already established for these strains in MoDC [84]. However, with the exception of CD80, BDC and Mo showed an even greater potential for bacterial upregulation of surface markers than MoDC, possibly because BDC and Mo in the unstimulated state are less mature than the in vitro-generated MoDC. The DC maturation marker, CD83, has been reported to be also a Mo-activation marker [167], and in our study it was upregulated on Mo by *E. coli* and *L. reuteri*. In a study by Karlsson et al. comparing bacterial stimulation of Mo and MoDC [162], low-level TNF-α and IL-6 production was induced in Mo, but not in MoDC, by two Gram-positive and two

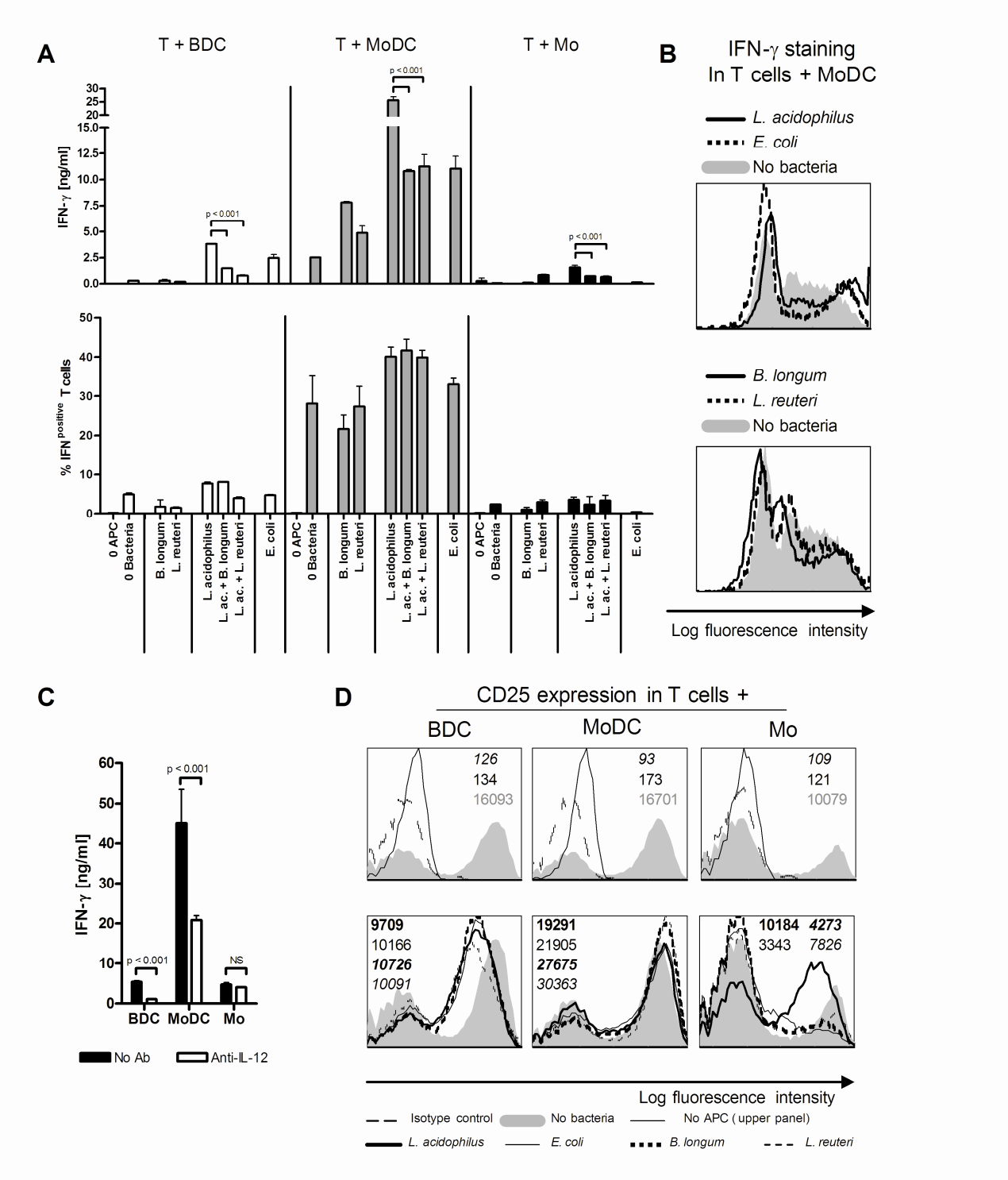

Figure 3.5: Naïve T cells are polarised to Th1 cells by specific gut-derived bacteria, independently of the type of APC. A. Naïve T cells were cultured with different types of allogeneic APC and 2.5 μg/ml gut-derived bacteria for 6 days. Bars (upper panel) depict mean supernatant IFN-γ concentration and SD of duplicate culture wells. Cells were re-stimulated, fixed, permeabilised and assayed for intracellular IFN-γ content by flow cytometry. Bars (lower panel) indicate the percentage of cytokine-positive

cells and SD of duplicate culture wells. B. Intracellular IFN-γ staining in T-cells cultured with MoDC with gut bacteria as indicated (solid and dotted lines) or without bacteria (filled histograms).C. Naïve T cells were cultured with allogeneic APC, *L. acidophilus* and anti-IL-12 antibody (10 μg/ml) for 6 days. Bars depict mean IFN-γ concentration and SD of duplicate culture wells. 'NS' indicates not significant. D. Expression of CD25 on T cells from the APC-T cell co-cultures. Numbers in grey indicate the geometrical mean fluorescence intensity of T cells cultured with APC and without bacteria. Numbers in italic indicate the geometrical mean fluorescence intensity of the isotype-stained cells and numbers in black the geometrical mean fluorescence intensity of T cells cultured without APC (upper panel only). In the lower panel, CD25 expression on T cells cultured with bacterially stimulated cells is compared to unstimulated cells with numbers indicating the geometrical mean fluorescence intensity of cells stimulated with individual bacterial strains (Bold: *L. acidophilus*, non-bold: *E. coli*, bold italic: *B. longum*, non-bold italic: *L. reuteri*).

Gram-negative strains, and IL-10 mostly by the Gram-negative strains. This is in accordance with our results in Mo, wherein *E. coli* induced the highest level of IL-10, and all strains induced some TNF-α and IL-6. However, both Mo and MoDC produced pro-inflammatory cytokines, and in response to more strains of bacteria than BDC. This may be due to their higher expression of TLR2 and TLR4, which for MoDC was higher in the present study than observed by Karlsson et al. [162]. Only in BDC and Mo, the modulation of TLR expression seemed related to APC function, as TLR2 was down-regulated in response to *L. reuteri* and *E. coli*; the same bacteria that upregulated CD40, CD83, TNF-α and IL-6 most potently in these APC. Modulation of TLR2 expression by *E. coli*, considered to be detected mainly by TLR4, was not unexpected, as TLR2 is involved in recognition of high doses of Gram-negative bacteria [168].

We observed very low levels of IL-12 p70 induced by *L. acidophilus* and *E. coli* in BDC and Mo compared to MoDC, but sufficient to induce IFN-γ production in autologous NK cells. In the study by Karlsson and colleagues [162], MoDC produced no IL-12 in response to Gram-positive bacteria except when primed with IFN-γ, which is contradictory to our findings. In our study, IL-12 was induced in 3-5 fold higher levels in all types of APC in the presence of exogenous IFN-γ (not shown), but the strain-dependent pattern of IL-12 induction was maintained. APC-induced IFN-γ production in NK cells was mediated essentially by IL-12, whereas CD69 expression was only weakly affected by blocking IL-12 (not shown). Of note, DC-derived IL-10 is not responsible for the inhibition of IL-12 by *L. reuteri* and *B. longum* leading to reduced IFN-γ responses in NK and T cells (Chapter 2). Bacterially stimulated MoDC were the strongest NK activators followed by Mo and BDC. The potent NK-stimulatory capacity of Mo is in accordance with Haller et al. [80], and it is likely that IFN-γ produced by the NK cells promoted IL-12 production in APC via a positive feedback loop enabling strong induction of IFN-γ in NK cells also by Mo, producing low amounts of IL-12 alone (Figure 3.3). This fact may also explain the apparent discrepancy

between particular LAB strains inhibiting IFN-γ production in NK and T cells but not inhibiting IL-12 in BDC or Mo, as undetectable differences in IL-12 levels may be amplified by IFN-γ during co-culture.

No induction of IFN-γ production or CD69 expression in NK cells was observed upon direct stimulation with any of the bacteria examined (not shown). Only Yun and colleagues [169] have observed a direct stimulatory effect of intact bacteria on highly purified NK cells using *Helicobacter pylori*. It may be that *Helicobacter pylori* interact with one or more of the TLRs shown to be functional in NK cells recognising bacterial products: TLR5 that binds flagellin [26] or TLR9 that detects unmethylated CpG motifs in bacterial DNA [27]. TLR2 and TLR4, which are believed to be involved in the recognition of LAB and LPS, respectively [162] are expressed at low levels in NK cells [170].

Both types of DC, immature or bacterially matured, and Mo cultured with *L. acidophilus* were capable of inducing activation of allogeneic naïve T cells as indicated by increased CD25 expression. We observed highly variable levels of IFN-γ production, with MoDC inducing the highest levels of IFN-γ, but the same bacterial strain, *L. acidophilus* X37, was the most potent IFN-γ-inducer in all APC-T cell cultures. The low IFN-γ production in response to DC and *E. coli* is consistent with the observations by Hafsi et al. [135]. As expected Mo were poorer at stimulating T cells than NK cells, due to their low expression of HLA-DR [165] and accordingly, Mo induced low amounts of IFN-γ in T cells, but the pattern of IFN-γ induction was comparable to the other types of APC. In a study of T cell polarisation by gut bacteria by Smits et al. [99], the MoDC employed were matured by TNF-α in addition to lactobacilli or *E. coli*. Under these conditions, *E. coli* is a strong inducer of IFN-γ-producing T cells, whereas lactobacilli do not increase the number of IFN-γ-producing T cells compared to TNF-α-treated MoDC. We observed a high number of IFN-γ-producing T cells with all allogeneic MoDC, but slightly more with *L. acidophilus*-matured DC, probably due to the unique IL-12-inducing properties of this *L. acidophilus* strain. The number of T cells after the co-culture did not differ between bacterial strains, but T cells stimulated with DC proliferated more than T cells cultured with Mo (not shown), which may also explain the lower IFN-γ response in Mo-T cell cultures. We also tested whether combining APC, NK cells and T cells with *L. acidophilus* induced an even stronger Th1 response, as NK-derived IFN-γ would promote polarisation of naïve T cells to Th1 cells, but this was not the case. Apparently, blood NK cells do not produce enough IFN-γ to amplify a Th1 response, whereas lymph node NK cells do [25, 171], and this may be of relevance in the mesenteric lymph nodes where DC carrying gut bacteria and NK cells meet.

MoDC matured in the presence of *L. reuteri* and *L. casei* induce IL-10 production in T cells and endow T cells with regulatory properties in the study by Smits et al. [99]. In our study, T cell derived

IL-10 was not detected in culture supernatants above levels produced by APC cultured alone, and intracellular IL-10 was measured and detected in very rare T cells without correlation to the stimulating bacteria employed. Hart et al. [172] combined naïve T cells and BDC with a mixture of probiotic strains, VSL#3, and did not observe any IL-10 or IL-4 production either, but fewer IFN-γ-producing cells with LAB than with LPS, as was also seen in our experiment with BDC and *B. longum* and *L. reuteri*.

In conclusion, we have observed differential induction of maturation markers and pro-inflammatory cytokines in Mo, MoDC and BDC, indicating different sensitivity to distinct intestinal bacteria. In contrast, although the amplitude of effector responses differed with MoDC being the most potent NK and T cell activators, the same bacteria promoted and inhibited IFN-γ responses when cultured with different APC. Primarily IL-12 and not TNF-α, IL-6 or co-stimulatory molecules was responsible for inducing IFN-γ in NK and T cells. This study provide strong evidence that the NK activating and Th1-promoting properties of gut-derived bacteria are conserved independently of type of APC, which simplifies the choice of in vitro model for characterisation of intestinal bacterial strains. The highly differentiated MoDC are the cells most responsive to bacterial stimuli and most potent activators of NK and T cells, while qualitatively resembling BDC, and they are therefore a sensitive APC model for studying bacterial immunomodulation, although they may not entirely reflect in vivo DC under the influence of tissue-derived factors.

Acknowledgements

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4. Dendritic cells isolated from gut-associated lymphoid tissues differ from spleen dendritic cells in their response to gut-derived bacteria

Preliminary data generated in collaboration with Sofie Toft Frisenette and Hanne Frøkiær.

Abstract

Commensal gut bacteria and probiotics have potent effects on the immune system, which are probably mediated by intestinal dendritic cells (DC). These bacteria differentially mature in vitro-generated DC and polarise subsequent T cell responses, but this kind of DC is thought to represent an inflammatory phenotype. In this study, we compared the response of mouse resident Peyer's patch (PP) DC, mesenteric lymph node (MLN) DC, and spleen DC to three strains of gut bacteria: *Bifidobacterium longum* Q46, *Lactobacillus acidophilus* X37 and *Escherichia coli* Nissle 1917.

Bacterial maturation of DC, measured as upregulation of CD40 and CD86, occurred independently of tissue origin, and *E. coli* was the strongest inducer of maturation. Expression of CCR7 and CD103 on the surface of DC, which is necessary for the induction of gut-homing regulatory T cells, increased along with the maturation markers. Cytokine patterns induced by the different bacteria and revealed by cytometric bead array (IL-6, IL-10 and TNF-α) were similar in spleen and MLN DC, and contaminants of these DC populations produced large amounts of IFN-γ in response to *L. acidophilus*. PP cells produced IL-6 only in response to *E. coli*, little IL-10 and no TNF-α or IFN-γ, and this low cytokine production was not due to inhibition by TGF-β. In contrast, *L. acidophilus*-induced secretion of IFN-γ and TNF-α by MLN cells was down-regulated by endogenous TGF-β

Commensal-induced responses of spleen and MLN DC resemble maturation of in vitro-generated DC, but MLN DC and PP DC cytokine responses differ markedly although they do upregulate surface maturation markers. In addition, MLN cells responded to bacterial stimulation with higher IFN-γ production than spleen cells. It therefore seems critically important for immune system polarisation whether MLN or PP cells primarily interact with commensal and probiotic bacteria.

Introduction

The human gastro-intestinal tract harbours 10^{13}-10^{14} bacteria, which implies that bacterial cells outnumber the body's own cell by a factor of 10 [173]. It is evident that these bacteria and the host mutually benefit from the intestinal environment. Intestinal bacteria possess enzymes permitting digestion

of food components that the human digestive enzymes cannot degrade, and thereby make nutrients available to the human cells. It is also well-established that short chain fatty acids produced by the intestinal flora maintain a milieu hostile to pathogens and induce epithelial cell proliferation improving the intestinal barrier [174]. However, an equally important property of the intestinal flora is to promote the early development of the host immune system and continually regulate it during life. We have previously studied the effect of selected members of the intestinal microbiota (strains of *Lactobacillus* and *Bifidobacterium*) on the immune cells predominantly responsible of immune system regulation, dendritic cells (DC), derived from human blood and from mouse bone marrow precursors, and shown that bifidobacteria and some lactobacilli polarise DC towards a regulatory phenotype, whereas other lactobacilli promote Th1-inducing cytokine production in DC [83, 84].

DC in the gut-associated lymphoid tissues (GALT) are present both in the subepithelial dome region (SED) and in the interfollicular regions (IFR) of PP, and scattered through the lamina propria connective tissue. SED DC are characterised by their expression of CD11b, whereas DC of the IFR express CD8. $CD8^{-}CD11b^{-}$ double-negative DC are present in both SED and IFR [54]. LP DC are predominantly $CD11b^{+}$ [175]. DC from LP continuously migrate to the MLN and PP DC migrate from the SED to the IFR, but also to MLN, as MLN DC contain distinct DC subsets with phenotypes corresponding to PP DC and LP DC [52]. GALT DC in general display an immature phenotype, and upregulation of CCR7, permitting migration to IFR and MLN, occurs in the absence of maturation [52]. MLN (and DC migration to MLN) are essential in the induction of 'oral tolerance' to fed protein antigens whereas PP are dispensable [176], suggesting that LP DC are responsible for uptake of soluble antigens [51].

PP and MLN also play a central role in controlling the interaction between the host immune system and commensal bacteria. DC carrying commensals, under normal conditions only penetrate as far as the MLN [67], and the systemic immune system probably remains ignorant of the intestinal microbiota [73]. Gnotobiotic (germ-free) mice possess fewer DC in MLN than mice with a normal intestinal microbiota [72], and furthermore these mice have reduced $CD4^{+}CD25^{+}$ Treg activity [68], indicating an important role for commensal microorganisms in balancing the immune system. PP is the main site of induction of IgA-producing plasma B cells by DC loaded with commensal bacteria [67]. It has been shown that also LP DC detect luminal bacteria by extending dendrites through the epithelial cell tight junctions [60, 177]. DC in the intestine are under the influence of suppressive tissue factors such as thymic stromal lymphopoietin (TSLP) possibly making them less responsive to stimulation by commensals [61]. In addition, human colonic DC express low levels of TLR2 and TLR4 compared to blood DC, but expression is increased in patients with IBD concomitantly with higher expression of maturation markers and increased production of

IL-12 and IL-6. However, recognition of commensals by TLR2 and TLR4 also plays a protective role in IBD [178]. LP DC and a subset of MLN DC express the α_E chain of the $\alpha_E\beta_7$ integrin, CD103 [53]. These DC are responsible for the induction of gut-homing CD8 T cells [53], and are also required for the recruitment of $CD4^+CD25^+$ Tregs to the intestine [50].

In light of the involvement of LP, PP and MLN in tolerance induction and immune regulation we were interested in the response of GALT DC to commensal bacteria. To mimic the effect of these bacteria in the intestinal environment we investigated how representative strains of lactobacilli, bifidobacteria and non-pathogenic *E. coli* interact with cell preparations enriched in DC from the PP, MLN and spleens of mice.

Materials and methods

Isolation of spleen, MLN and PP DC, and spleen NK cells

Balb/c mice, aged 8-10 weeks, were killed by cervical dislocation. Spleen and MLN were removed, cells released by squeezing in Hank's balanced salt solution, calcium and magnesium free (CMF-H, Cambrex Bio Science, East Rutherford, NJ), and the cell suspension passed through a cell strainer. Small intestines were excised and the contents expelled. PP were excised, mechanically disrupted in CMF-H and single cells separated from tissue using a cell strainer. PP tissue was digested for 30 min. at 37°C in RPMI 1640 (Cambrex Bio Science) with 0.2 Wünsch units of Liberase Blendzyme 2 (Roche Applied Science, Indianapolis, IN), and liberated cells were pooled with the mechanically released cells. DC were isolated from spleen, MLN, and PP cell suspensions with the murine $CD11c^+$ cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched DC populations contained 30-60% $CD11c^+$ DC. Bonemarrow-derived DC (BMDC) were generated as previously described [83]. Splenic NK cells were isolated with the murine NK cell isolation kit (Miltenyi) and 70% of isolated cells were positive for the NK cell marker CD49b. Cells were cultured in a humidified 5% CO_2 atmosphere in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Cambrex Bio Science) referred to as complete medium.

Stimulation with intestinal bacteria

The bacterial strains used for stimulation of DC were *Lactobacillus acidophilus* X37, *Bifidobacterium longum* Q46 and *Escherichia coli* Nissle 1917. *L. acidophilus* X37 and *B. longum* Q46 were isolated from intestinal biopsies and typed at Department of Food Science, University of Copenhagen, Denmark.

Bacteria were grown overnight in de Man, Rogosa and Sharpe broth (*L. acidophilus* and *B. longum*) or in Luria-Bertani broth (*E. coli*) both from Merck (Darmstadt, Germany), washed twice in sterile PBS, resuspended in PBS and UV-irradiated for 15 min. Suspensions of *L. acidophilus* and *B. longum* used in cell culture experiments contained < 0.015 endotoxin units/ml. Bacterial suspensions were diluted on a dry matter basis in complete medium prior to use in cell culture experiments. For stimulation, DC were plated at $2 \cdot 10^5$ cells/well in 200 µl complete medium in 96-well round-bottomed plates. Cells were stimulated with 10 µg/ml of UV-killed intestinal bacteria. To some wells, anti-IL-12p70 antibody or anti-TGF-β1 antibody was added (10 µg/ml, R&D Systems). NK cells were cultured at 10^5 cells/well with or without $5 \cdot 10^4$ cells/well of DC-enriched cells and 10 µg/ml of UV-killed intestinal bacteria. From all cultures, supernatants were harvested after 18 hours and replaced with PBS containing 1% FCS and 0.1% NaN_3 before staining of the cells with fluorochrome-labelled antibodies.

Surface marker and cytokine analysis

DC and cells contaminating the DC preparations were characterised using the following antibodies: anti-CD4-allophycocyanin (BD Biosciences, Franklin Lakes, NJ), anti-CD11c-phycoerythrin (PE, BD Biosciences), anti-CD11c-allophycocyanin, anti-MHCII-PE, anti-CD40-PE, anti-CD103-PeCy7, anti-CCR7-PE (eBioscience, San Diego, CA), anti-CD8-allophycocyanin, anti-CD80-PE, anti-CD86-PE (Southern Biotech, Birmingham, AL) and anti-CD49b-allophycocyanin (Miltenyi). Prior to antibody staining, cells were incubated with 5 µg/ml anti-CD16/CD32 (BD Biosciences) at room temperature for 10 min. Cytokines in the supernatant were measured with the cytometric bead array (CBA) Mouse Inflammation Kit (BD Biosciences), with sensitivities as indicated: IL-6: 5 pg/ml; IL-10: 17.5 pg/ml; IL-12p70: 10.7 pg/ml; TNF-α: 7.3 pg/ml; IFN-γ: 2.5 pg/ml. TGF-β1 was quantified using DuoSet ELISA (R&D Systems, Minneapolis, MN).

Results

Isolation of GALT DC and spleen DC

Cells of MLN, PP and LP were compared to spleen cells as regards their expression of CD4, CD8, the NK marker CD49b and the DC marker CD11c (Table 4.1). LP was relatively rich in DC, but the number of cells obtainable was very low. Therefore, we conducted stimulation experiments with DC isolated only from spleen, MLN and PP, to assess whether the response of these DC populations to commensal bacteria was qualitatively comparable to the results obtained in murine bone-marrow derived DC and human blood DC [83, 84]. DC were enriched to 30-60%, with the highest purity in spleen cell suspensions

and lowest in PP. Contaminating cells included T cells and NK cells (Table 4.1). Less than 0.5% of the NK cells expressed CD11c (not shown). Isolated $CD11c^+$ DC from all tissues displayed an immature phenotype (Figure 4.1, upper row); with MLN DC expressing slightly higher levels of CD40 and CD86 than spleen DC and PP DC. BMDC were included as a reference, because we have previously shown that lactobacilli are capable of maturing BMDC [83].

Table 4.1: GALT and spleen cell populations in Balb/c mice (n = 3-5). Numbers in italic indicate the percentage of a given cell type in cell suspensions enriched for DC from different tissues in one representative experiment with spleen cells pooled from two animals and MLN and PP cells pooled from eight animals.

	Cells isolated per mouse	***% $CD11c^+$ cells (SD)***	***% $CD4^+$ cells (SD)***	***% $CD8^+$ cells (SD)***	***% $CD49b^+$ cells (SD)***
LP	App. $5{\cdot}10^5$	5.3 (0.9)	14 (1.7)	1.7 (1.2)	1.2 (0.9)
PP	App. $5{\cdot}10^6$	1.7 (0.6)	23 (4.1)	3.3 (1.2)	0.77 (0.5)
PP DC		*30*	*7.4*	*5.4*	*1.6*
MLN	App. 10^7	1.1 (0.2)	52 (3.4)	16 (4.5)	0.97 (0.6)
MLN DC		*52*	*17*	*11*	*1.8*
Spleen	App. 10^8	2.0 (0.5)	24 (3.2)	9.2 (3.0)	4.9 (0.3)
Spleen DC		*59*	*15*	*9.2*	*1.1*

Gut-derived bacteria mature DC independently of tissue origin, but regulate CCR7 and CD103 differently in DC from PP, MLN, and spleen

Culture of all types of DC overnight without stimuli increased their maturation level substantially (Figure 4.1). Addition of *B. longum* or *L. acidophilus* further increased expression of the co-stimulatory molecules, CD40 and CD86, to a similar extent in all DC, whereas culture with *E. coli* even more potently increased the expression of these maturation markers (Figure 4.1).

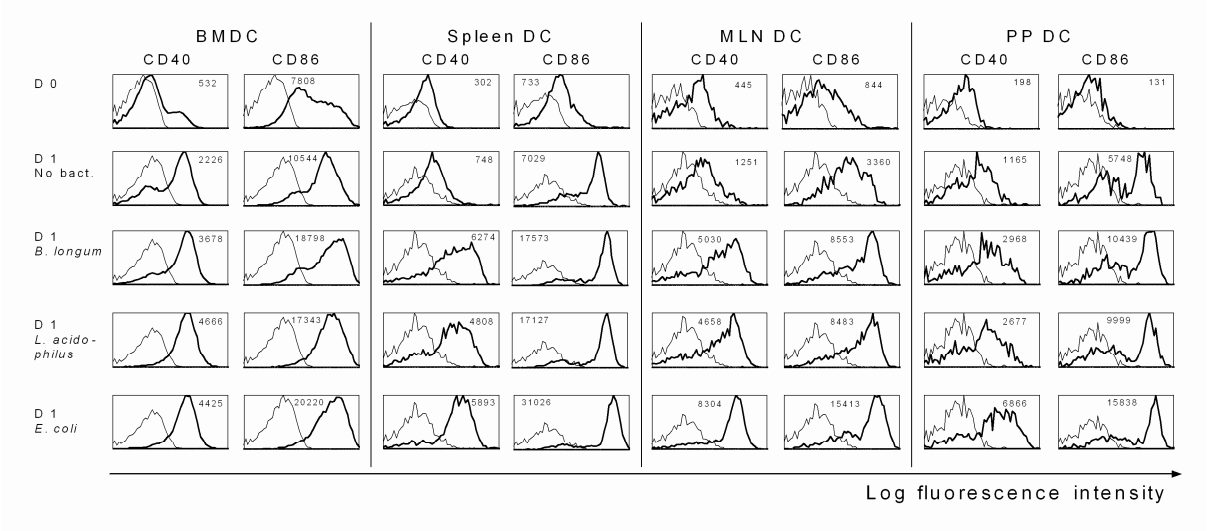

Figure 4.1: Gut-derived bacteria mature DC. DC were generated from bone-marrow precursors or isolated from spleen, MLN and PP and stimulated with single strains of UV-killed intestinal bacteria ($2 \cdot 10^5$ cells/well in 200 µl, 10 µg/ml bacteria). Expression of CD40 and CD86 was detected on $CD11c^+$ dendritic cells before (D0) and after 18 hours of culture (D1). Thin lines indicate cells stained with an isotype control antibody and bold lines indicate staining with antibodies specific for the indicated surface marker. Numbers indicate the geometrical mean fluorescence intensity of specifically stained cells. Results from one experiment of four independent experiments with cells pooled from eight mice are shown.

In addition to the maturation surface markers, we studied the expression of CD103 and CCR7 on DC from spleen, MLN and PP (Figure 4.2). CD103 is present on a subset of MLN DC, but almost absent from freshly isolated spleen DC [53]. Expression of CD103 on PP DC has not been described previously, but it was low on the majority of freshly isolated PP DC. All DC decreased their expression of CD103 during overnight culture, although for the MLN and PP DC it seemed that the expression on the initial $CD103^+$-population decreased, while expression of CD103 on $CD103^-$-cells increased slightly. Induction of CD103 expression by bacteria has never previously been studied, but we detected a minor increase in CD103 expression of spleen DC upon bacterial stimulation. In MLN DC, CD103 expression was noticeably increased when DC were stimulated overnight with bacteria compared to medium alone. This seemed to only be case for PP DC, which were CD103 negative prior to stimulation, resulting in no increase in mean fluorescence intensity of these cells.

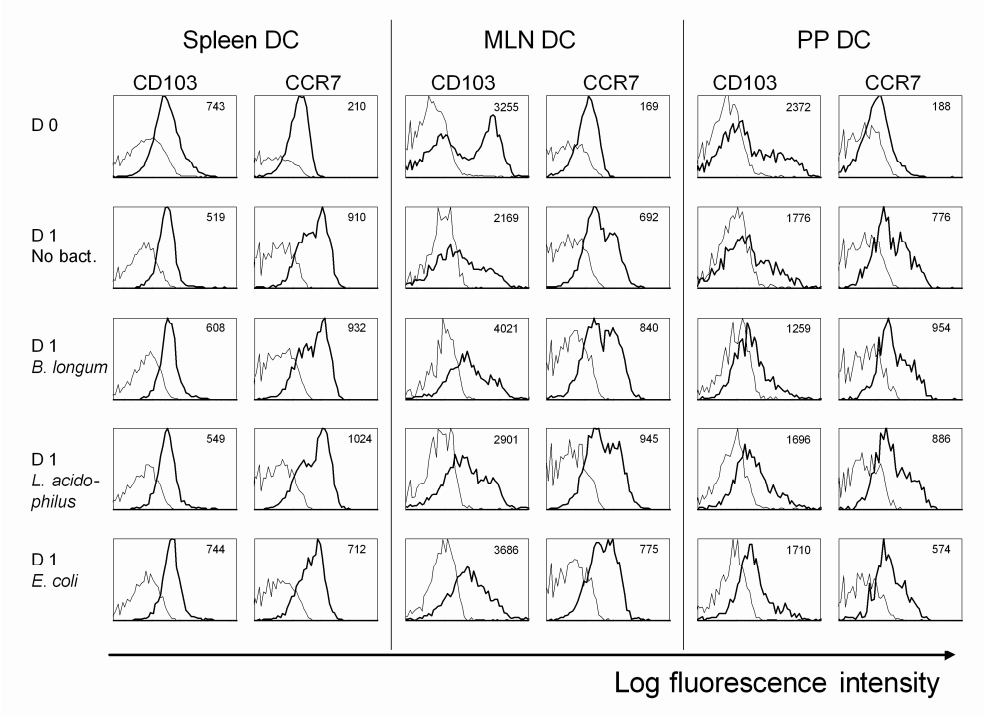

Figure 4.2: Gut bacteria differentially induce CCR7 and CD103 on DC from spleen, MLN and PP. DC were isolated from tissues by positive magnetic separation and stimulated with single strains of UV-killed intestinal bacteria ($2 \cdot 10^5$ cells/well in 200 µl, 10 µg/ml bacteria). Expression of CCR7 and CD103 was detected on $CD11c^+$ dendritic cells after 18 hours of culture using specific fluorochrome-labelled antibodies. Thin lines indicate cells stained with an isotype control antibody and bold lines indicate staining with specific antibodies. Numbers indicate the geometrical mean fluorescence intensity of specifically stained cells. Results from one experiment representative of four independent experiments with cells pooled from eight mice are shown.

In freshly isolated cells, CCR7 was expressed to a similar extent on all DC, and the expression increased during overnight culture in the absence of stimuli. Stimulation with UV-killed *E. coli* down-regulated the expression of CCR7 in splenic DC and PP DC, whereas culture with *B. longum* and *L. acidophilus* slightly increased CCR7 expression on DC. In MLN DC, all bacteria upregulated CCR7 expression, but *B. longum* and *L. acidophilus* more than *E. coli*.

Commensal bacteria induce production of IL-10, IL-6, TNF-α and IFN-γ in spleen and MLN DC preparations, but only IL-6 production in enriched PP DC

The cytokine response to gut bacteria was dependent on both cell origin and bacteria (Figure 4.3A). IL-6 was induced in all types of DC, especially by *E. coli* and to a minor extent by *B. longum* and *L. acidophilus*. IL-10 was induced in spleen and MLN DC by *B. longum* and *E. coli*, whereas PP DC did not consistently produce IL-10. TNF-α was produced by bacterially stimulated DC with particular bacteria inducing more TNF-α according to the scheme: *L. acidophilus* > *E. coli* > *B. longum,* except in PP DC,

wherein all bacteria induced low level TNF-α production. In all tissue-derived DC supernatants, TNF-α concentrations were more than 10 times lower and IL-6 concentrations more than 100 times lower than in BMDC, whereas IL-10 levels were only slightly lower than in BMDC (not shown). IFN-γ was also induced in enriched DC populations by *L. acidophilus,* probably due to contaminating T cells and/or NK cells, as IFN-γ production was absent in BMDC containing no lymphocytes. In all DC preparations, *L. acidophilus* were the major IFN-γ-inducing bacteria. IL-12p70 production was not increased above background levels of 30-100 pg/ml by bacteria (not shown), presumably because IL-12 was consumed by IFN-γ producing cells. Intracellular detection of IFN-γ was attempted to identify the IFN-γ producing cell type, but was complicated by the fact that DC non-specifically stained positive for IFN-γ production, irrespectively of tissue origin and stimulus (not shown). TGF-β was produced mainly by MLN and PP DC independently of bacterial stimulus (Figure 4.3B), and we hypothesised that TGF-β was responsible for suppressing cytokine levels in the PP DC supernatants. However, cytokine production by PP cells did not increase when a TGF-β neutralising antibody was added, whereas production of all cytokines, especially TNF-α and IFN-γ increased in MLN cells when TGF-β was blocked (Figure 4.3C).

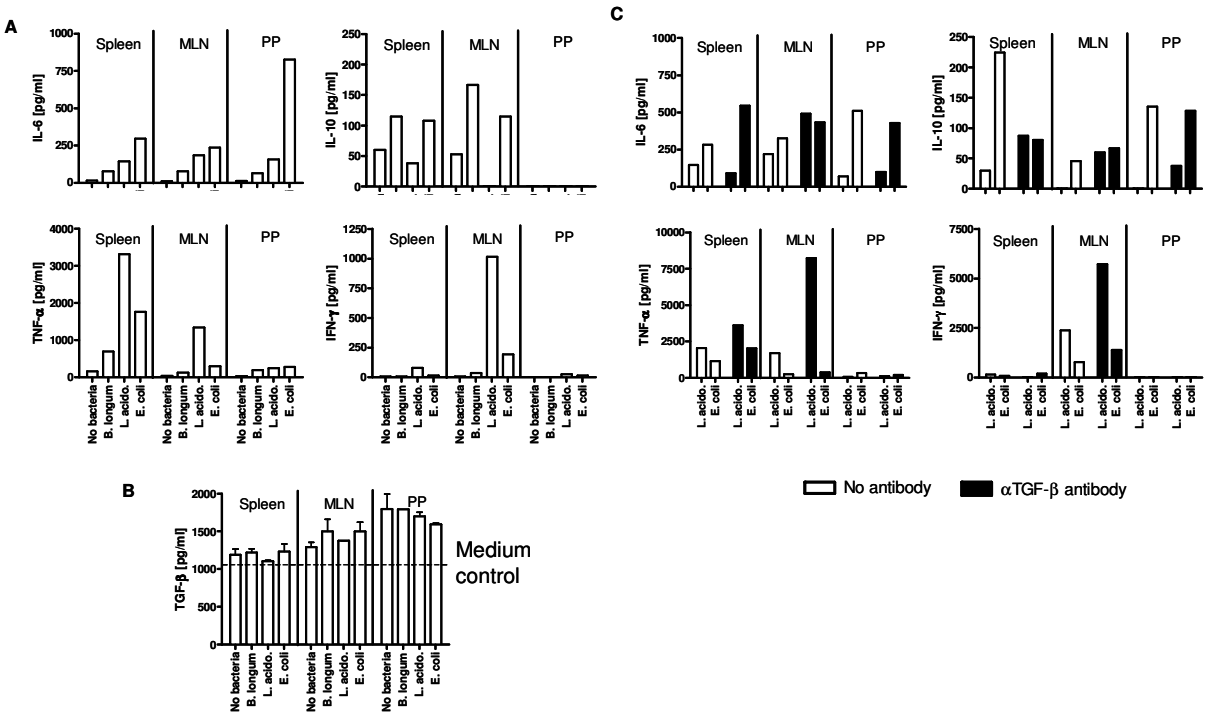

Figure 4.3: Gut bacteria induce production of different cytokines in cell suspensions enriched in DC from spleen, MLN and PP. DC were isolated from tissues by positive magnetic separation and stimulated with single strains of UV-killed intestinal bacteria ($2 \cdot 10^5$ cells/well in 200 μl, 10 μg/ml bacteria). A. IL-6, IL-10, TNF-α and IFN-γ in cell supernatants were quantified after 18 hours of culture by CBA. Due to low number of cells, data are single determinations. B. TGF-β in the DC cultures was quantified by ELISA. Data are means and SD of duplicate samples. The dotted line indicates the TGF-β content in samples of

cell-free complete culture medium. C. DC were cultured as above but in the absence (white bars) or presence (black bars) of 10 μg/ml anti-TGF-β1 antibody. IL-6, IL-10, TNF-α and IFN-γ in cell supernatants were quantified after 18 hours of culture by CBA. Results are representative of four independent experiments, each with cells pooled from eight mice.

In other DC and DC-NK cell systems, we have observed that TNF-α, IFN-γ and IL-6 production induced by *L. acidophilus* is potently reduced by simultaneous addition of bifidobacteria ([83, 84] and Chapter 2). In this experiment this was only the case in DC-enriched spleen cells, wherein TNF-α, IFN-γ and IL-6 production induced by *L. acidophilus* and *B. longum* added together was lower than the level induced by *L. acidophilus* alone (Figure 4.4). No substantial inhibition of *L. acidophilus*-induced cytokine production by *B. longum* occurred in MLN cells, although this difference between spleen and MLN DC in susceptibility to inhibition was only statistically significant for IL-6 across four independent experiments. IL-10 was induced at intermediate levels by *L. acidophilus* and *B. longum* in combination.

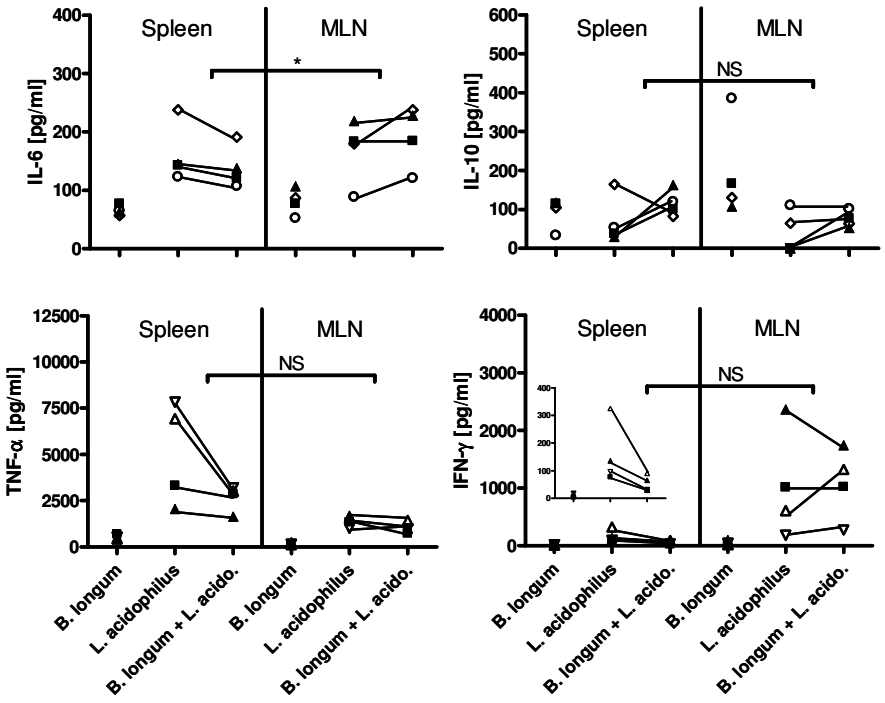

Figure 4.4: IL-6, IFN-γ, and TNF-α production induced in spleen and MLN suspensions by *L. acidophilus* is reduced when *B. longum* are added together with *L. acidophilus*. DC were isolated from tissues by positive magnetic separation and stimulated with either UV-killed *B. longum*, *L. acidophilus* or both ($2 \cdot 10^5$ cells/well in 200 μl, 10 μg/ml of each strain of bacteria). Cytokines in cell supernatants were quantified after 18 hours of culture by CBA. Due to low number of cells, data are single determinations. * indicates that the *difference* between IL-6 production elicited by *L. acidophilus* alone or combined with *B. longum* is significantly different between spleen and MLN ($P < 0.05$). For the other cytokines this difference is not significant. Different symbols indicate four independent experiments with cells pooled from eight mice. Insert shows IFN-γ production in splenic cells on a smaller scale.

IFN-γ production in response to gut bacteria is reduced upon blocking of IL-12 and may be attributable to NK cells

We were interested in determining whether NK cells were responsible for the IFN-γ production seen in cell populations from spleen and MLN enriched for DC. This would be probable as we have seen that human DC cultured with certain UV-killed gut bacteria induce IFN-γ production in purified blood NK cells (Chapter 2). Characteristic for the IFN-γ production induced in NK cells by bacterially stimulated DC is the dependence on IL-12, but this also applies to induction of IFN-γ production in Th cells. We tested whether addition of an IL-12 neutralisation antibody reduced cytokine production induced in enriched DC populations by *L. acidophilus* and *E. coli*. Indeed, IFN-γ production was reduced by more than half by an anti-IL-12 antibody (Figure 4.5A), whereas IL-6, IL-10 and TNF-α production were not affected (not shown). We next compared the IFN-γ-production induced by *L. acidophilus* and *E. coli* in DC with the response of spleen NK cells cultured with the same enriched DC from spleen and MLN (NK/DC ratio 2). IFN-γ was produced primarily in MLN cells enriched in DC in response to *L. acidophilus*, but to the same extent in spleen NK cultured with spleen DC and MLN DC. PP cells enriched in DC did not produce IFN-γ and did not induce IFN-γ production in spleen NK cells either. IFN-γ was consistently reduced also in the NK-DC co-cultures when IL-12 was neutralised. *E. coli* induced IFN-γ in MLN DC, but not in spleen NK cells cultured with MLN DC. In a parallel co-culture experiment with spleen NK cells and BMDC we observed that *L. acidophilus* X37, when used to stimulate BMDC, induced 10 times more IFN-γ in spleen NK cells than *E. coli* (Figure 4.5B).

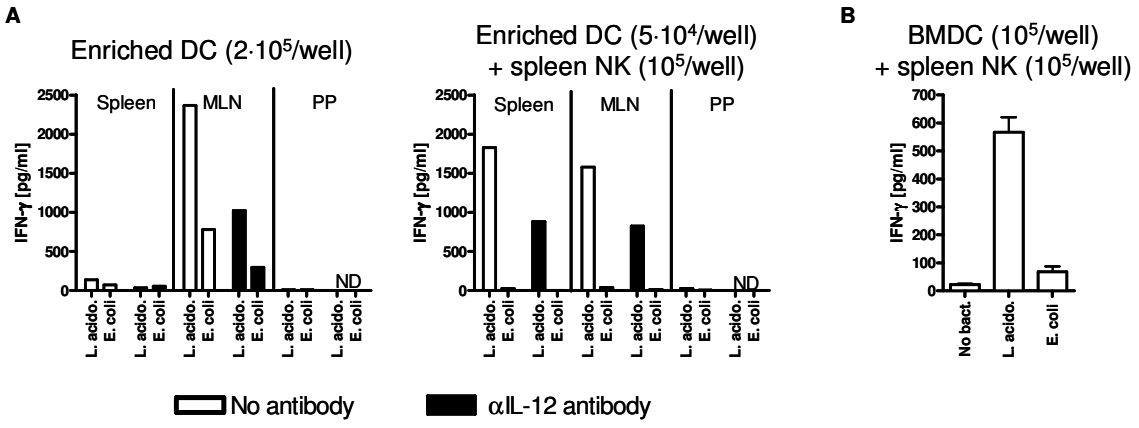

Figure 4.5: A. IFN-γ production induced by gut bacteria in cell suspensions enriched in DC differs from the production induced in DC co-cultured with NK cells. DC were isolated from tissues by positive magnetic separation and stimulated with single strains of UV-killed intestinal bacteria ($2 \cdot 10^5$ DC/well in 200 μl, 10 μg/ml bacteria, left), or co-cultured with negatively selected splenic NK cells and stimulated with intestinal bacteria ($5 \cdot 10^4$ DC/well and 10^5 NK/well in 200 μl, 10 μg/ml bacteria, right). Anti-

IL-12p70 antibody was added where indicated. IFN-γ in cell supernatants was quantified after 18 hours of culture by CBA. Due to low number of cells, data are single determinations. 'ND' indicates not done. B. IFN-γ production induced by gut bacteria in NK cells co-cultured with BMDC. DC were pre-stimulated for one hour before addition of NK cells (10^5 DC/well and 10^5 NK/well in 200 μl, 5 μg/ml bacteria) IFN-γ in cell supernatants was quantified after 18 hours of culture by ELISA.

Discussion

Maturation of DC isolated from different tissues was induced by all intestinal bacterial isolates tested, with *E. coli* being the most potent at inducing maturation. We have observed before that *E. coli* are more potent at inducing DC maturation due to the high stimulatory potential of *E. coli* LPS relatively to the stimulatory components of lactobacilli and bifidobacteria; peptidoglycan, lipoproteins and lipoteichoic acid [84]. The extent of maturation of MLN and PP DC induced by intestinal bacteria was comparable to maturation of LP DC, which in the presence of LPS upregulate maturation markers more than overnight culture alone [51]. In contrast, rat lymph DC migrating from the intestine towards MLN do not mature upon stimulation with LPS [179]. CCR7, the chemokine receptor enabling migration in response to CCL19 and CCL21 predominantly present in T cell areas of lymph nodes and PP, has been described to be a maturation indicator in BMDC, but is only marginally increased in maturing spleen DC [180]. Iwasaki & Kelsall [54] have reported upregulation of CCR7 on PP DC in response to CD40 ligation. This is in accordance with our findings, where maturation of spleen DC only marginally increased (with Gram-positive bacteria) or reduced (with Gram-negative bacteria) CCR7 expression, whereas maturation of MLN and PP DC by Gram-positive bacteria was accompanied by a substantial increase in CCR7 expression. This may indicate a different priming of MLN and PP DC enabling their migration in response to these bacteria and thus points towards Gram-positive commensal bacteria having a specific role in immune homeostasis in the gut. This responsiveness may apply in a general fashion in the intestine, as the organisation of isolated lymphoid follicles (smaller PP-like structures) is influenced by both CCR7 and the presence of bacteria [44]. Steady-state migration of LP DC carrying apoptotic epithelial cells (containing antigens) to MLN occurs in the absence of DC maturation [52] and in germ-free animals [136], but this does not preclude a role for bacterially induced CCR7 in the interplay between host and commensals.

We showed for the first time that also the expression of CD103 on DC is modulated by Gram-negative and Gram-positive bacteria. CD103 expression was increased along with maturation especially on the initially $CD103^-$ subset of MLN DC. $CD103^+$ DC induce gut-homing $CD8^+$ $CCR9^+$ T cells [53], and recruit CD4+CD25+ regulatory T cells to the intestine [50]. It is therefore likely that intestinal commensals contribute to the tolerogenic environment of the intestine by inducing CD103 expression on

DC. CCR7 is indispensable for migration of $CD103^+$ DC to MLN and for induction of gut-homing T cells [53]. We observed concomitant upregulation of CCR7 and CD103 on DC stimulated with commensal Gram-positive bacteria, but it remains to be shown whether the high level of CD103 expression seen in LP DC [53] is a consequence of bacterial stimulation.

We observed major differences in the cytokines secreted in response to bacteria by DC from different tissues. Ex vivo, DC from the human colon LP produce IL-10 but no IL-12 in response to enteroinvasive *Salmonella* [61] and the VSL#3 mixture of eight probiotic LAB [172]. Mouse colonic LP DC respond to both LPS and *B. longum* with a modest IL-12 production, and only *B. longum* induce IL-10 production [181]. We did not manage to obtain pure LP DC populations, but both MLN and PP cells enriched for DC also produced mainly IL-10, and little IL-12 and IFN-γ in response to *E. coli* and *B. longum*, and less IL-10 along with more IFN-γ in response to the Gram-positive *L. acidophilus*.

TGF-β was produced in the different DC preparations regardless of stimulation, but we could confirm that TGF-β produced by PP cells exceeded the amount produced by MLN cells, which again was superior to the TGF-β secretion of splenic cells [182], and the opposite was the case for TNF-α, demonstrating the anti-inflammatory environment in the MLN. Interestingly, blocking of TGF-β had a marked effect on bacteria-induced cytokine production only in MLN cells. The absence of TNF-α and IFN-γ production in bacterially matured PP DC preparations, even when TGF-β was neutralised, may indicate the absence or non-responsiveness of contaminant cell types producing these cytokines, as PP DC are considered potent inducers of T cell IFN-γ [183]. Upon in vivo stimulation with particularly *E. coli*, the TGF-β produced by MLN and PP cells would be present concomitantly with increased amounts of IL-6, possibly leading to the induction of ThIL-17 cells, speculated to play a benign role in the intestinal environment increasing barrier function, but contributing to autoimmune reactions elsewhere in the body [5]. IL-6 induced by *E. coli* may therefore represent alertness of the immune system towards this commensal, and in addition promote synthesis of protective secretory IgA [57]. IFN-γ produced by MLN cells in response to both intestinal bacteria, on the other hand, may recruit Th1 cells, and together with TGF-β, support development of oral tolerance [184].

Remarkably, IFN-γ was produced to the greatest extent by stimulated MLN cells enriched for DC, and MLN cells released IFN-γ not only in response to *L. acidophilus* as spleen cells, but also in response to *E. coli*. These differences in IFN-γ production may be assigned to the different amounts of NK and T cells contaminating the DC preparations from the two tissues, or to the inherent properties of these NK and T cells. As the proportions of T and NK cell contaminants in the spleen and MLN

preparations were comparable (Table 4.1), likely, NK cells of MLN were more prone to produce IFN-γ than spleen NK cells, as has been shown for human lymph node NK cells [24]. The finding that the inhibition of *L. acidophilus*-induced IFN-γ production by *B. longum* shown to govern the interaction between human DC and NK (Chapter 2), did not occur in MLN cells may also reflect the stronger cytokine producing potential of MLN NK cells. MLN NK cells may overcome inhibition, maybe by being very sensitive to IL-12. We were unable to identify the cell type responsible for IFN-γ production in the cell populations enriched for DC, but as the IFN-γ production occurred already within 18 hours, NK cells are the major candidates, possibly assisted by NKT cells or the interferon-producing DC population with cytotoxic potential recently described [41]. IFN-γ production was reduced upon neutralisation of IL-12, but this does not rule out any cell type possibly implicated. In any case, this early IFN-γ response to intestinal bacteria especially in the MLN may play a role in the important skewing of the intestinal immune response away from Th2 responses and towards Th1 responses, which takes place in early life.

In conclusion, the finding that MLN and PP DC show different responses to commensal bacteria compared to spleen DC, indicate that the interaction between commensal bacteria and selected DC subsets is highly specialised and may play a role in tolerance induction in the gut. DC at different sites have a different subset distribution, and this may contribute to the apparent “division of labour” between tissues observed here, but also different tissue determinants may affect DC responses. We show that NK cells respond readily to stimulation by bacteria in the presence of DC, but with different outcomes dependent on the tissue origin of both DC and NK cells. Therefore, the Th1 polarising effect of different groups of commensal bacteria may depend on the sites of their encounters with the immune system.

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5. Summarising discussion

The present chapter summarises similarities and differences between the immunomodulatory potential of selected gut-derived bacterial strains studied in different APC and the resultant effector responses of NK cells and T cells.

The growing interest in NK cell biology and especially their expression of TLRs, which may recognise LAB, was the initial focus of the work of the present thesis, but we realised that direct stimulation of NK cells by LAB did not occur in the absence of accessory cells. Instead, NK cells were potently stimulated by LAB in the presence of MoDC and by MoDC pre-incubated with LAB as shown in Chapter 2. The cross-talk between LAB-matured MoDC and NK cells resulted in increased NK cell proliferation and cytotoxicity regardless of the LAB strain, and in IFN-γ production when IL-12-inducing LAB were used. Maturation of MoDC by LAB caused upregulation of CCR7 and HLA-I, and protected them from killing by NK cells, a mechanism which may preserve DC to stimulate/regulate NK cells and other cells of the MLN and the systemic immune system in vivo.

Human MoDC are a commonly used model of human tissue DC, but as they are propagated in the presence of the cytokines IL-4 and GM-CSF, they may differ from naturally occurring DC. In Chapter 3, we chose to compare LAB-stimulation of MoDC to peripheral blood DC to validate the concept that induction of cytokines in DC and NK cells by LAB is highly strain-dependent. In this analysis, blood Mo were also included, as they are considered progenitors of DC, and Mo have previously been shown to induce cytokine secretion in NK cells when present together with LAB [80]. Mo and MoDC have been shown by others to respond differently to Gram-negative and Gram-positive bacteria [162], so the probiotic and gut-derived *E. coli* strain Nissle 1917 was included as a reference Gram-negative strain. While MoDC cultured with LAB remained the most potent cytokine-inducers in NK cells, Mo and blood DC displayed a similar strain-dependent pattern of IL-12-induction in DC and IFN-γ in NK cells and T cells. Blood-derived APC, however, responded with different amounts of pro-inflammatory cytokines to specific LAB strains when compared to MoDC. This could reflect the alertness of blood APC to bacterial components, which probably changes with their differentiation into tissue DC.

To approach the actual site of interaction between the immune system and the gut flora we wished to study the cytokine and maturation response to LAB and *E. coli* of DC from the mouse GALT. The passage from the human to the mouse system was possible as members of the group have already established that the response to LAB and Gram-negative bacteria (LPS) in human MoDC and in mouse BMDC is very similar [83, 84]. So far we have been unsuccessful in obtaining sufficient DC from the LP of

mice to conduct in vitro stimulation experiments, but it was possible to compare cells enriched in DC from PP and MLN to spleen DC (Chapter 4). Interestingly, PP DC seemed to respond differently from MLN and spleen DC to LAB and *E. coli*, whereas only minor differences were seen between the MLN and spleen DC, in particular regarding their response to stimulation with *B. longum*. For the first time, we showed that LAB and *E. coli* differentially modulate the expression of the CD103 molecule, specific for DC with the ability to imprint gut-homing T cells. Our preliminary data also suggest that NK cells from MLN possess a stronger IFN-γ-producing potential than spleen NK cells. This would be in accordance with findings in human lymph nodes, which contain primarily $CD56^{bright}$ cytokine producing NK cells [24].

It is clear from this thesis that the previously shown capacity of different strains of LAB to induce varying levels of IL-12 production in DC has consequences for effector cell responses, as LAB together with APC induced IFN-γ to a different and strain-dependent degree in NK cells and T cells. We tested only a limited number of gut-derived strains as the DC-stimulatory capacity of different bacterial strains permits grouping of LAB and *E. coli* strains into three major groups [84]: 1) lactobacilli, which induce high amounts of IL-12 in DC, 2) other lactobacilli, but primarily bifidobacteria, which induce low amounts of IL-12 in DC, and 3) *E. coli* strains, which induce a high degree of maturation in DC but low amounts of IL-12 and whose effect resembles the effect of LPS.

The *L. acidophilus* strain X37 represents the first-mentioned lactobacilli, and these bacteria potently induced DC-dependent IFN-γ production in both NK cells and T cells. *L. acidophilus*-stimulated Mo were also capable of inducing IFN-γ-production in autologous NK cells, but not in allogeneic T cells, probably due to lack of co-stimulatory molecules. When *L. acidophilus* was used to stimulate murine cell populations enriched in DC, IFN-γ was induced in a partially IL-12-dependent way in both spleen and MLN cells, whereas PP cells did not respond to stimulation with *L. acidophilus*. It is unknown how many commensal gut lactobacilli have properties similar to *L. acidophilus* X37, and whether the effect of these bacteria is counter-balanced by other bacteria in vivo, but it is likely that Th1-promoting bacteria participate in the maturation of the immune response. The Th1-polarising properties of *L. acidophilus* X37 and similar strains may also be exploited as oral adjuvants or “nutraceuticals”, as Th1 cells inhibit the propagation of Th2 and ThIL-17 cells involved in allergy and autoimmune diseases, respectively. Administration of *L. acidophilus* X37-like bacteria could also be beneficial in the protection against cancer, as Th1 cells promote cytotoxic T cells responses and counteract the action of regulatory T cells, which can be promoted by cancer cells [21]. NK cell-derived IFN-γ is assumed to play a major role in the induction of Th1 responses in lymph nodes [25]. We were unable to show that IFN-γ produced by NK cells in response to *L. acidophilus* X37-stimulated DC amplified the IFN-γ response induced in T cells

by the same DC, but this could be because we studied blood NK cells, the majority of which does not potently secrete cytokines. Our preliminary data on MLN cells stimulated with *L. acidophilus* X37 suggest that lymph node NK cells indeed have a larger ability to secrete IFN-γ and this may affect the polarisation of GALT T cell responses.

B. bifidum S13.1, *B. longum* Q46 and *L. reuteri* DSM12246 are poor IL-12-inducing LAB, which when mixed with *L. acidophilus* before being used as a DC stimulus, inhibit IL-12 production [84]. This inhibition of IL-12 production in MoDC caused inhibition of IFN-γ production in NK cells and T cells. Furthermore, *B. bifidum* S13.1 and *L. reuteri* DSM12246 modulated the expression of activation markers on NK cells. Interestingly, the IL-12-inhibitory activity of bifidobacteria was reduced in blood DC and the inhibitory activity of *L. reuteri* was low in Mo, which may indicate a different PRR repertoire in these cell types. Indeed, TLR2 and TLR4 were expressed to a different extent in all APC tested in Chapter 3, but it has not yet been possible to link the IL-12 inhibitory action of certain bacteria to specific PRRs. In agreement with the findings in human cells, *B. longum* Q46 induced lower levels of cytokine production in mouse spleen and MLN DC than *L. acidophilus* X37, but inhibition of *L. acidophilus*-induced IFN-γ production was only pronounced in splenic cells. It is not yet known why mouse MLN DC may resemble blood DC in this respect.

Consistent with the human intervention studies showing an increase in NK cell activity after administration of *L. rhamnosus* GG [113], *L. casei* Shirota [125] and *B. longum* HN019 [127], which are LAB strains that induce rather different cytokine profiles in APC [185, 186], we observed increased proliferation and cytotoxic activity in human NK cells stimulated by DC matured by both *B. bifidum* S13.1, *L. reuteri* DSM12246 and *L. acidophilus*. We did not study the DC cytokines or cell-to-cell interactions responsible for these NK effector functions, as we assumed they resembled DC factors induced by non-LAB bacteria. However, it would be interesting to identify and isolate the LAB component responsible for the increment in NK cell cytolytic activity and proliferation, as this component would have potential therapeutical uses, e.g. as a stimulus for DC used in cancer immunotherapy.

The *E. coli* Nissle 1917 strain differs from the LAB strains in that it stimulates DC when used at much lower concentrations [84]. *E. coli* strains induce a high degree of expression of HLA-DR and co-stimulatory molecules on APC, but rather low levels of IL-12 production [84]. In consequence, *E. coli*-matured DC did not induce high levels of IL-12-dependent IFN-γ production in mouse or human NK cells, but intermediate levels in human allogeneic T cells, wherein cell-to-cell contact presumably plays a larger role in activation. *E. coli* LPS is a commonly chosen stimulus when immune function is studied, but we have shown here that the Th1-inducing potential of *E. coli* was inferior to *L. acidophilus* both in NK cells

and T cells, at least when high doses of bacteria were used. In contrast, *E. coli* induced rather high levels of IL-6 and IL-10 in all tested APC. IL-10 induces Tregs [187], whereas IL-6 abrogates Treg regulation and promotes ThIL-17 cell differentiation when present together with TGF-β [188, 189]. It may be that the downstream pathways of TLR2 and TLR4 are divergent in a way that causes ligation of TLR2 to initiate IL-12 production, whereas ligation of TLR4 predominantly results in production of cytokines with complex regulatory properties. This hypothesis, however, is probably too simple, as both Gram-negative and Gram-positive bacteria interact with more than one TLR and likely also other PRRs.

We did observe similar strain-dependent properties of the selected gut-derived bacteria in all cell systems tested, except for PP DC, which validates the in vitro-generated DC as a useful model, e.g. for studying signalling events and effector cell responses induced by gut-derived bacteria. However, much is still to be learned about the uptake and processing of commensal microorganisms in the GALT. Therefore, individual bacterial strains (delivered orally as probiotics, or already an established part of the gut microbiota) studied in vitro must be confirmed to have a beneficial effect in vivo, preferably in animal models of allergy, autoimmune diseases or IBD. As some bacterial strains counteract the Th1-promoting effect of other strains, it would also be of interest to know the distribution of bifidobacteria and lactobacilli in the intestine, longitudinally and axially, to investigate whether these types of bacteria or bacteria-derived components are in simultaneous contact with the immune system.

Conclusions

- Strain-specific interactions between LAB and MoDC have profound effects on the cross-talk between DC and autologous NK cells, including consistent induction of NK cell cytotoxicity and proliferation and IL-12-dependent upregulation of IFN-γ production in NK cells.

- LAB and *E. coli* induce strain-specific cytokine production in blood DC and Mo, and IFN-γ production in NK cells co-cultured with these APC is induced in an IL-12-dependent fashion. However, bacterially matured MoDC remains the most potent cytokine inducers in NK cells.

- LAB and *E. coli* differentially activate allogeneic T cells through priming of MoDC, blood DC and Mo, and the capability of specific bacteria to induce IFN-γ production in T cells resembles their IFN-γ-inducing potential in NK cells.

- Mouse spleen DC respond to gut-derived bacteria with a pattern of cytokine production resembling MoDC, whereas MLN DC and PP DC react differently, indicating the importance of the route of uptake of commensal and pathogenic gut bacteria.

- Bacterial stimulation of all cell types tested revealed strain-dependent properties of LAB, and these can potentially be exploited in diseases such as allergy, autoimmune diseases and cancer, wherein immunomodulation towards Th1 or Treg would be beneficial.

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